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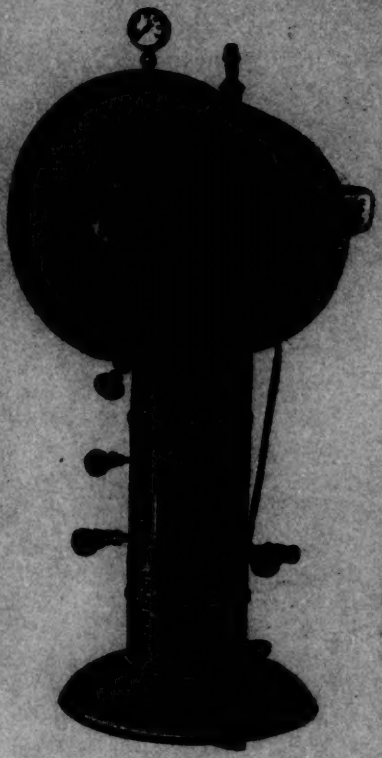

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The *Journal* requests that, when possible, authors prepare their manuscripts in conformity with these suggestions.

STUDIES ON POLLEN AND POLLEN DISEASE.*

I. THE CHEMICAL COMPOSITION OF RAGWEED POLLEN.

By JESSIE HORTON KOESSLER.

(From the Hull Laboratories of Biochemistry and Pharmacology, University of Chicago, Chicago.)

(Received for publication, December 7, 1917.)

INTRODUCTION.

The search for determining the fraction of the pollen substance in which is inherent the active principle evoking the well known symptoms of pollen disease, or hay fever, suggested a prior investigation of the chemical composition of pollen with special consideration of its nitrogen distribution.

Considering the corresponding part taken by microorganisms in the causation of any infectious disease, some explanation must be offered why a knowledge of the chemical composition of pollen might be of aid in understanding the disease caused by it. For it is generally true that the chemical analysis of the dead body substance of any pathogenic microorganism in all its nitrogenous and non-nitrogenous fractions, has failed to throw much light on the mechanism of its pathogenicity and the immunity reaction evoked in the organism. Yet, in some instances, as in tuberculosis, a certain gain has been derived for our understanding of the peculiar life and reaction problems of the tubercle bacillus, by a chemical study of its body substance. The etiological agent of

* This work was suggested by Dr. Koessler. Preliminary work on the nitrogen distribution of ragweed pollen had led him to suspect that the figures obtained by Kammann on the protein content of rye pollen would not hold for ragweed pollen. For a detailed discussion of the immunological aspect of pollen disease see Koessler, K. K., The specific treatment of hay fever (pollen disease), in Forchheimer, F., Therapeusis of Internal Diseases, New York, 2nd edition, 1915, v, 671.

This work was done prior to June, 1917, and written up before the article by Frederick W. Heyl, which appeared in July, 1917, was read.

the pollen disease differs from any microorganism causing an infectious disease in several important respects. The individual life history of the pollen cell is ended on entering the human organism; it does not multiply after invasion of the host like microorganisms of bacterial nature. Pollen, being a cell of most definite function in the plant organism, ends its living functions on entering the human tissues and, therefore, is unable to produce a toxin as a secretion product of its normal metabolism. It must be conceded, at least on theoretical considerations, if not from clinical evidence, that the poisonous action which pollen exerts on certain individuals may have its source in a preformed poison of similar nature to the glucoside of *Rhus toxicodendron* (poison ivy).

The results of early investigators, as John in 1814, and Braconnot in 1829, only brought out the fact that different pollen strains vary considerably in chemical composition. Analyses of ragweed pollen have not been published and the only others of interest in this connection are the results published by Kammann on rye pollen. Kammann's work was done at the instigation of Dunbar, who, in 1903, reiterated the statement first made by Blackley in 1873, that hay fever is a disease due to plant pollen, and not of bacterial origin. Kammann found that the alcohol-ether extracts are irritating to the conjunctiva of all persons, normal as well as those suffering from hay fever, but that the albumin portion of the protein is the portion giving a specific reaction in the hay fever patient only, and, therefore, is the toxic portion causing hay fever. In Germany the usual form of hay fever is the June cold which is apparently due chiefly to grass pollen. Dunbar had considered the starch granules the toxic portion of the pollen grain until he found that certain pollen strains (ragweed and goldenrod), which do not contain starch granules, also cause hay fever. Kammann's analysis of rye pollen is given below.

	per cent	
Water	10.18	
Ash	3.4	
Inorganic substance	13.58	
Organic "	86.42	
Alcohol-ether extract	3.00	Organic substance.
Carbohydrate	25.00	
Non-protein nitrogen	18.00	
Protein	40.00	

Study of Ragweed Pollen.

The pollen grains of *Ambrosia artemisifolia* and *Ambrosia trifida* are small and round. The outer membrane is very thick

and presents a rough, spike-like exterior. Treated under the microscope with chromic acid, everything dissolves except a reticulum of very resistant material, which, in other varieties of pollen, is known as cutin. This is supposed to be of a fatty or waxy nature. The very thick walls of ragweed pollen are probably composed of this substance. If the pollen is treated with concentrated H_2SO_4 a gradual weakening of the wall takes place at three equidistant points with subsequent formation of a three-lobed structure, suggesting the normal lobular appearance of the apple and pine pollen. Lugol's solution stains the whole pollen yellow but does not bring out any dark blue, rod-like bodies, indicating the presence of starch.

Extraction by Alcohol and Ether.

The pollen was extracted alternately with hot alcohol and ether in the Landsiedl apparatus with frequent changes of the solvent and until completely exhausted. The results varied somewhat in three samples of *Ambrosia trifida* extracted, but, as

TABLE I.

Sample.			
	gm.	*gm.*	*gm.*
Pollen taken	2.58	31.16	7.68
	per cent	*per cent*	*per cent*
Alcohol-ether soluble	43.10	38.4	
" " " nitrogen	0.87		0.69
" " " phosphorus	0.19		
" " -insoluble	58.7	61.6	
" " " nitrogen		4.65	4.63

given in Table I, show an average of about 40 per cent of alcohol-ether-soluble substance, which is a much higher figure than that obtained from any pollen recorded.

Carbohydrate.—The total carbohydrates (polysaccharides and simple sugars) were determined by the acid hydrolysis according to Bulletin No. 107[1] and the Bertrand[2] modification of the Munson-

[1] *U. S. Dept. Agric., Bureau of Chemistry, Bull. 107*, 1912, 241.

[2] Bertrand, G., *Bull. Soc. chim.*, 1906, xxxv, 1288.

Walker method for reducing sugars. This gave 6.89 per cent calculated as glucose. The pollen grains of the residue appeared entirely unchanged under the microscope; after a second acid hydrolysis no more sugar was obtained. Another sample of pollen was boiled with 15 per cent HCl for 15 minutes. Here too, the pollen grains looked entirely normal under the microscope and the sugar test was therefore not made. Even on digestion with trypsin at 37° for 24 hours, the pollen gave no evidence of disintegration of its very resistant walls.

In another sample of pollen, the polysaccharides were first freed from the fat and protein by treatment with alkali, and then when subjected to acid hydrolysis gave only 3.9 per cent reducing sugar. It was noticed on boiling the pollen with hydrochloric acid that the condenser and neck of the flask showed a violet discoloration which indicates loss of sugars in this hydrolysis, due to furfural formation.

Distribution of Nitrogen.

Total Nitrogen (Kjeldahl-Gunning) in Dry Pollen.—The following figures were obtained on three samples of pollen from entirely different sources.

	per cent	per cent
Ambrosia trifida (*a*)	4.48	4.89
" " (*b*)	4.72	4.75
" *artemisifolia*	4.49	4.94
Average	4.72	

Extractive and Lipin Nitrogen.—The analyses reported in Table I show that 0.69 to 0.87 per cent of nitrogen is extracted by hot alcohol and ether. Roughly speaking, this may be called lipoid and extractive nitrogen.

Extraction by 5 Per Cent NaCl Solution and the Distribution of Nitrogen in the Various Fractions and Subsequent Extraction by Boiling 75 Per Cent Alcohol.

2.703 gm. of dried pollen were ground for 1 hour with 2 gm. of pumice which had been previously ground, washed, and ignited. The mixture was then shaken for 15 minutes at room temperature with 100 cc. of 5 per cent NaCl solution, and then centrifuged.

The supernatant liquid was decanted onto a quantitative filter and the extraction repeated four times. The filtrates were combined and diluted to 500 cc. with distilled water. Portions were taken for various estimations. The insoluble residue of pollen and pumice was next extracted four times with 50 cc. portions of 75 per cent alcohol. The results of these experiments, as well as the nitrogen distribution, after extraction of the original pollen with 95 per cent alcohol, are here tabulated:

	Nitrogen.	Per cent of total nitrogen.
	per cent	
Total	4.73	
Soluble in 5 per cent NaCl solution	1.89	39.9
" " 5 " " NaCl " and coagulable	0.10	2.11
" " 5 " " NaCl " " non-coagulable	1.66	35.09
Soluble in 75 per cent alcohol after 5 per cent NaCl extraction	1.57	33.2
Final insoluble residue	1.35	28.5
Total	4.81	
Soluble in hot 95 per cent alcohol and ether	0.62	13.1
Insoluble " " 95 " " " " "	4.15	87.7
Total	4.77	

Extraction of Nitrogenous Substances and Their Separation.

In the above experiments, in order to extract the proteins, the pollen was ground for 2 hours with sea-sand before extraction. We have since found out that the grinding has no effect and that an equally high per cent of nitrogen is obtained by extraction with 8.5 per cent NaCl solution without previous grinding. The NaCl extract gives strong protein reactions. Only the biuret test is unavailable, due to the intense yellow of the extracted pigment. The residue also gives protein reactions, but much weaker. Kammann, who has done the most recent work on pollen chemistry, states that the nitrogen of the residue consists of unsaturated compounds. The nitrogen of the NaCl extract was 1.89 per cent and might be due to extractives, amino-acids, amines, and to albumins, globulins, and other proteins, soluble in salt solution.

The residue, after the above extraction, was extracted with 75 per cent alcohol. This extract yielded 1.57 per cent nitrogen and might contain prolamines, extractives, amines, amino-acids, salts, and lipoids. The insoluble residue, containing any albuminoids, phosphoproteins, or nucleoproteins, gave 1.35 per cent nitrogen. The sum of the nitrogen content in these three fractions made 4.81 per cent, a figure slightly larger than the total nitrogen.

Further study was done on the NaCl extract only, this being the fraction containing the most activity and nitrogen, and generally the most protein.

Following the work of Kammann[3] on rye pollen, we extracted 100 gm. of pollen (wet weight) with 300 cc. of NaCl solution at 37°, for 10 hours. Instead of using 5 per cent NaCl solution, we used 8.5 per cent, as Osborne has shown that a 10 per cent NaCl solution is more effective in extracting protein than weaker solutions. The pollen residue was pressed out by means of the Buchner press, 265.5 cc. being recovered. The extract was very concentrated and so highly colored as to be nearly olive-green. The xanthoproteic, Millon, and ninhydrin reactions were positive, and the extract was physiologically active when tested on the conjunctiva of hay fever patients. The nitrogen content was 1.82 per cent, calculated to the original pollen (dry weight).

The NaCl extract was then precipitated with eight times the volume of 95 per cent redistilled alcohol. Kammann used absolute alcohol for this precipitation, but the difference in results owing to this change would be small. In place of his white, finely granular powder, our precipitate was black and sticky and was equal to 7.5 per cent of the pollen weight.

The alcoholic precipitate contained only 0.318 per cent nitrogen calculated to the original pollen or, in other words, only about 2 per cent protein if all is protein nitrogen. It gave very positive results by the xanthoproteic, Millon, and ninhydrin tests. The yellow color, even in high dilutions, obscured the biuret test. Kammann records the rye pollen to contain 40 per cent protein, but whether all could be thus extracted and then precipitated by alcohol is a question.

[3] Kammann, *Beitr. chem. Physiol. u. Path.*, 1904, v, 348–351.

The filtrate from the alcoholic precipitate was also too yellow to show the biuret reaction. The xanthoproteic and Millon's tests were negative, but the ninhydrin test was fairly positive. The Kjeldahl nitrogen in it was 1.32 per cent of the pollen. It was not physiologically active.

Protein Hydrolysis of Pollen.

The protein hydrolysis was carried out according to the method of Van Slyke,[4] after the pollen had first been thoroughly extracted with absolute alcohol and ether in the Landsiedl apparatus. During the hydrolysis, which lasted 20 hours, the contents of the flask became a dark brownish red, and along the neck of the flask and in the condenser, a purplish red color developed, as in the case of the acid hydrolysis for the polysaccharides. Black insoluble particles appeared after several hours boiling and increased as hydrolysis proceeded. This humic substance, or melanoidin, according to Osborne and Jones, Gortner and Blish[5] and White[6] is, no doubt, a mixture of secondary decomposition products formed from different split products of the protein on acid hydrolysis. Studies by White in this laboratory agree in general with the results obtained by Gortner and Blish and also indicate that glucosamine, tyrosine, and cystine may be involved in the formation of the melanoidin fraction in protein hydrolysis.

As the quantity of humin was so large in the pollen hydrolysis, it had to be filtered off before the next step in the analysis could be taken, and the nitrogen determined separately. The large amount of melanoidin nitrogen obtained, together with the fact that the hydrolysis had to be carried out in presence of the carbohydrates and in presence of other unknown substances, should be taken into consideration in interpreting the results of this hydrolysis. The cystine, amido, and non-amino nitrogen figures probably are not correct for these reasons.

The results of the protein hydrolysis are given in Table II.

[4] Van Slyke, D. D., *J. Biol. Chem.*, 1911–12, x, 15; 1915, xxii, 281.

[5] Gortner, R. A., and Blish, M. J., *J. Am. Chem. Soc.*, 1915, xxxvii, 1630.

[6] White, E. H., The reactions of amino-acids with carbohydrates and strongly acid solutions, Master's thesis in Physiological Chemistry, University of Chicago, 1915.

TABLE II.

Hydrolysis of Ragweed Pollen.

Nitrogen.				N in dry pollen.	
		Corrected.	Total.		
	gm.	*gm.*	*per cent*	*per cent*	
Total	0.3701	0.3701	100	4.65	
Total minus humic	0.3404	0.3404	91.97	4.28	
Humic	0.0297	0.0397	8.02	0.37	
Amido	0.0243	0.0243	6.56	0.30	
Arginine	0.0513	0.0545	14.72	0.68	Arginine in pollen = 2.13 per cent.
Histidine	0.0482	0.0520	14.05	0.65	Histidine in pollen = 2.41 per cent.
Cystine	0.00276	0.0053	1.43	0.06	Cystine in pollen = 0.57 per cent.
Lysine	0.0113	0.0118	3.18	0.14	Lysine in pollen = 0.97 per cent.
Amino of bases	0.0430	0.0482	13.02	0.60	
Total " "	0.1136	0.1237	33.42	1.56	
Amino of filtrate	0.1489	0.1437	38.82	1.81	
Non-amino of filtrate	0.0325	0.0276	7.46	0.34	
Total of filtrate	0.1814	0.1713	46.28	2.16	

The sum of the amido nitrogen and the total nitrogen of the bases plus the total nitrogen of the filtrate equals 4.03 per cent. The total nitrogen of pollen minus the humic nitrogen was found to be 4.28 per cent, showing there was only 0.25 per cent nitrogen unaccounted for. The fact that 73.30 per cent of the total nitrogen in the ether-alcohol-extracted pollen is accounted for by the diamino-acid nitrogen plus the amino nitrogen in the filtrate therefrom, is very good evidence that probably most of the nitrogen in the alcohol-extracted pollen is present in protein combination. The most striking feature of this hydrolysis is, probably, the fact that although the absolute per cent of histidine is not high, relatively the per cent of histidine is unusually high as compared with that of arginine. Van Slyke found the per cent of arginine and histidine equal in gliadin, and in phosphorescent infusoria histidine is reported to be twice that of arginine. In all other cases the arginine is from two to six times that of histidine, or even much higher.

Histidine is of much interest in this connection due to the possibility of the production from it of imidazolylethylamine. This amine is highly toxic and produces all the symptoms of anaphylaxis. The hay fever toxin is said to be an anaphylatoxin resulting from the parenteral digestion of the proteins of the pollen, the pollen protein in this way acting as a sensitizer, and as a poison, by entering the circulation of individuals subject to hay fever.

The results of my chemical analysis of ragweed pollen may be summarized as follows:

		per cent
Inorganic		21.1
Moisture	10.5	
Ash	10.6	
Organic		78.9
Total reducing sugars after hydrolysis	6.89	
Ether-soluble lipoids	10.3	
Fatty acid after hydrolysis	4.75	
Phytosterol* (Windaus method)	0.34	
Insoluble in ether, but soluble in 95 per cent alcohol	12.5	
Extractives, etc., soluble in alcohol (resins) and water	11.5	
Insoluble residue (crude fiber, proteins, etc.)	37.71	
Total	100.0	100.0

* An attempt was made to identify this by the acetate method of Kerr (*U. S. Dept. Agric., Bureau of Animal Industry, Circ. 212*, 1913) but it was not possible to free the crystals from resinous impurities.

Distribution of Nitrogen, Phosphorus, and Sulfur.

	Moist pollen.		
	Nitrogen.	Phosphorus.	Sulfur.
	per cent	*per cent*	*per cent*
Total values	4.72	0.66	0.63
Soluble in hot 95 per cent alcohol and ether	0.62	0.17	
Insoluble " " 95 " " " " "	4.15	0.40	
Soluble in 5 per cent NaCl	1.89		
Extracted next by 75 per cent alcohol	1.57		
Insoluble residue left	1.35		

No accurate method has as yet been developed to differentiate quantitatively the protein from the non-protein nitrogen. The NaCl solution does not extract all the proteins, as glutelins, prolamines, phosphoproteins, and albuminoids would remain almost entirely in the residue. In one experiment, the nitrogen of the NaCl extract was 1.89 per cent of the weight of the pollen. On extracting the residue of pollen with 75 per cent alcohol, 1.57 per cent alcohol-soluble nitrogen was found and the residue from this extraction yielded 1.35 per cent nitrogen. We cannot say how much of this nitrogen was of protein character. In our study of the NaCl extract in the last experiment, we found 1.82 per cent nitrogen in this fraction. This multiplied by 6.25 gave 11.37 per cent as the highest possible protein figure. Probably this is not all protein. On being precipitated with eight volumes of alcohol, which precipitates most proteins, the precipitate yielded only 0.318 per cent nitrogen, or a possible protein content of only 1.99 per cent in the pollen. The filtrate from this precipitate gave 1.32 per cent nitrogen, or 8.25 per cent as the maximum protein content. It would appear then, either that the protein in ragweed pollen is small in amount, or that it is present to a large extent in a form non-extractable by NaCl solution and possibly also very incompletely precipitated by alcohol.

The results of the protein hydrolysis of the ether-alcohol-extracted pollen show a relatively high amount of arginine and histidine nitrogen in the material and probably these are present in protein combination in the original pollen. The results also indicate that probably all of the nitrogen in the alcohol-ether-extracted pollen is present in protein combination.

I wish to express my thanks to the Upjohn Company of Kalamazoo and to Dr. A. Parker Hitchins, Director of the Mulford Laboratories, for the material used in this work. I am indebted to Miss Sophia H. Eckerson for assistance in studying the form and resistant membranes of the pollen by microchemical tests.

In conclusion, I wish to express my deep appreciation of the invaluable help given me in this work by Dr. Fred C. Koch.

EXPERIMENTAL SCURVY OF THE GUINEA PIG IN RELATION TO THE DIET.*

By BARNETT COHEN and LAFAYETTE B. MENDEL.

(From the Sheffield Laboratory of Physiological Chemistry in Yale University, New Haven.)

(Received for publication, July 1, 1918.)

In 1895, Theobald Smith reported that guinea pigs died of a peculiar hemorrhagic disease when fed on a cereal diet without succulent vegetables. More than 10 years later, Holst and Frölich directed attention to the similarity between this malady in the guinea pig and human scurvy. Whether the etiological factor is identical in both these syndromes is a point that still remains to be definitely settled. However, there are many points of resemblance between the two, since the clinical pictures are practically alike, except that affection of the gums is not usually found in the guinea pig. It has also been suggested that swelling of the joints in the same animal might be ascribed to a condition of rickets (Gerstenberger, 1918).

It has long been recognized that scurvy is a nutritional disease, for numerous authors testify that its appearance was preceded by a monotonous diet of some peculiar nature. Also, though the pathogenesis is still rather obscure, the means for the cure of this malady were known for a long time: a change in diet to fresh food—especially green vegetables—would initiate a prompt improvement and speedy cure.

In the minds of observers in the earlier studies of the subject infection and toxemia played a prominent rôle as the causative agent. Later, with the advent of the vitamine theory to explain deficiency diseases, it was assumed that the absence of an antiscorbutic substance in the dietary was responsible for the nutritional failure. More recently, owing to the claims of McCollum and his coworkers, the existence of an antiscorbutic vitamine is questioned. Indeed, they deny that scurvy is a deficiency disease in the true meaning of the term. They ascribe it to some defect in the *texture* of the diet; that is, unsatisfactory physical factors induce a chain of circumstances which result in damage to the wall of the alimentary canal and a consequent toxemia.

It is now generally agreed that an exclusive diet of cereals or other plant seeds will cause a failure of nutrition of the guinea pig with disturbances

* Preliminary reports of these experiments were made to the Society for Experimental Biology and Medicine, April 17 and May 15, 1918.

that are very much like the human type of scurvy. This has been shown by Holst and Frölich, Jackson and Moore, McCollum and Pitz, and a host of others. However, Farnam (1916), working with a large number of animals in this laboratory, was unsuccessful in producing the disease regularly. This divergence from the generally reported results still remains unexplained. A set of our animals placed under conditions closely comparable to those in her experiments developed the characteristic clinical picture as anticipated.

The present work was undertaken with two main objects in view: first, to ascertain whether experimental scurvy is demonstrable at will in the guinea pig; and second, to investigate some of the dietary aspects of the problem in the light of recently developed theories.

Symptomatology.

The experiments here reported in the production and cure of experimental scurvy were made with one species of laboratory animal, the guinea pig; and it must therefore be kept in mind that our remarks and conclusions apply indirectly and only by analogy to the comparable syndrome in other species. In view of the present uncertain state of the problem, it seems desirable that we describe as clearly as possible what is here understood to be experimental scurvy from the clinical standpoint.

In the guinea pig this is a disease the onset of which is usually characterized first by a tenderness of the joints; and in our experience, the wrists, ankles, and knees become involved in the order named, though there are many exceptions. There follows in a day or so, a gradual swelling of the affected joints, often to twice or three times the original diameter of the bone. Sometimes, in the younger animals, a joint will fracture spontaneously,—the wrist being most susceptible in this regard. The older animals develop a difficulty in using their hind legs, which seem to become stiffened or paralyzed. In order to relieve the pain in the affected member, the animals will lie on their side or back and assume the scurvy position described by Chick and Hume (1917). If curative measures are instituted early enough, the swellings disappear entirely; if not, these knobs harden into exostoses that can be felt through the skin. The disease appears also to affect the junctions of the ribs with the cartilages, and in advanced cases we have been able to palpate these costochondral enlargements described by Jackson and Moore (1916). The animals winced when these were touched.

Accompanying the joint enlargement, one notices a marked hypersensitiveness that is succeeded by a dulness or lethargy. The symptoms de-

scribed may appear while the animals eat well and gain in weight steadily. Then follows a loss in appetite with a resulting decline in weight to death.

Hemorrhage of the gums in scorbutic guinea pigs has been reported by McCollum and Pitz, and Jackson and Moore have found it in a few of their animals. However, in common with Holst and Frölich and with Gerstenberger, we have never observed this feature in our animals. Very rarely (in four cases), there was an appearance of submucous hemorrhage at the base of the lower incisors. Oftener (in ten or fifteen cases), there was observed a hyperemia or congestion at the same site.

Loosening of the teeth was quite a common occurrence in the animals with scurvy. Occasionally during life, the lower incisors were shown to be loose. Sometimes they would break off, though never in normal pigs or in animals with scurvy that was recently developed. At autopsy the lower molars were nearly always loose,—at times to such a degree as to permit the teeth to be removed easily with forceps. The upper molars on the contrary were not so often affected in this manner.

For the sake of completeness, attention may be called to another manifestation that is said to be characteristic of the disease under study, though we have not had the facilities to investigate it. That is the x-ray picture of the affected joints. As first shown by Fraenkel,[1] there appears at the junction of the epiphysis and diaphysis of the long bones a white line which recedes very slowly after other symptoms have gone.

At autopsy, the two most noticeable conditions are hemorrhage and fragility of the bones. Our findings coincide in this regard with the descriptions given by Jackson and Moore. The viscera often were normal in appearance; though now and then the stomach, intestines, or cecum showed congestion, hemorrhage, or ulceration. These would occur usually on the oat and water or oat and milk diet, and less frequently on the soy bean diet.

Special attention was paid to the condition of the cecum which, according to McCollum and Pitz, may be the seat of the symptom complex known as scurvy. As a basis for comparison we had the autopsies of over a hundred guinea pigs, many of them normal, and the rest dead from conditions other than scurvy or intestinal disturbance. Except in the case of inanition, we have never observed one in which the cecum was not full or partly full of semifluid feces, this being the natural condition for the guinea pig. In our scorbutic animals, the cecum varied widely in appearance and character of contents, even in animals on the same diet. As a rule, those on the oats and water diet showed a larger incidence of impaction of feces in the cecum; and often

[1] Quoted by Gerstenberger, 1918.

damage to the wall was evidenced by congestion and hemorrhage. This was practically always the case in our animals fed with oats and milk. However, it did not occur nearly so often in the scorbutic animals when the symptoms were produced with the soy bean diet to be described below. Summarizing our experience with nearly one hundred scorbutic animals, we conclude that actual impaction of the feces in the cecum occurred in about one-quarter of the cases; and visible damage to wall, *i.e.* congestion or hemorrhage, or impaction, or both, was found in perhaps half of the cases. It should be noted that this statement covers all the diets we have tried.

Susceptibility of Animals at Different Ages.

The time of onset of the symptoms depends on the age of the animal as well as the nature of the diet. Young animals weighing from 110 to 250 gm. are best adapted to the production of the characteristic symptoms and lesions of scurvy. There are, however, limiting factors that should not be disregarded. If the diet is poor in protein or inorganic salts, the young animals may succumb so rapidly that the scorbutic symptoms may not have an opportunity to manifest themselves. This is particularly true of the monotonous cereal diet like oats. In such a case, older animals weighing about 350 gm. are more suitable. Taking this into consideration, we have found, other things being equal, that the younger the animal the quicker will the symptoms appear. Holst and Frölich who used cereal diets mainly, state that young animals weighing 150 to 200 gm. are not so suitable because these die too soon on such diets. These authors preferred animals weighing 300 to 550 gm. McCollum and Pitz state: "There is such great variation in the vitality of guinea pigs as shown by their reaction to experimental diets that uniform and consistent results cannot be expected in work of the character [they described]." This has not been our experience. We have found the guinea pig to lend itself to as consistent results here as have been reported for other animals in experiments of the same nature. Even in old, mature animals, we have succeeded in producing scorbutic symptoms, except of course that it took much longer and the physical condition of such animals was accordingly much worse.

Response to treatment was also remarkably consistent if begun in proper time. It may well be, however, that the variations found by McCollum and Pitz were due to the oat and milk diet used in their experiments. This will be made somewhat clearer in our discussion of the outcome of comparable experiments.

General Procedure and Methods.

All of the animals were under observation for a week or more before being started on a diet; and were always in good health at the beginning of the experiments. No epizootics have occurred though there were a few respiratory affections, mainly in the animals weakened by scurvy. Where possible, young or half-grown animals weighing 110 to 350 gm. were used. The older ones ranged in weight up to about 600 gm.

The cages were of metal with removable trays at the bottom. Except where otherwise noted, each tray contained a sheet of $\frac{1}{8}$ inch asbestos overlaid with small mesh galvanized wire screening. The trays were cleaned at least twice weekly, and where necessary daily. At such times the asbestos sheets were removed and heated until practically all the organic matter was burnt away. This arrangement provided a very satisfactory bedding for the animals and at the same time assured us of the fact that none of it was eaten. For a short time before this plan was evolved some of our animals consumed some of the asbestos. Before an experiment was begun and thereafter every 3 or 4 weeks, the cages were sterilized for 1 hour with live steam.

Except in a few cases noted, distilled water was supplied to the animals. The water containers were paraffined and were washed daily. At first, the animals on the milk diets were fed by means of a pipette, but this was found extremely impractical. The following arrangement proved satisfactory. A porcelain dish was firmly fixed in each cage and the requisite amount of milk placed in the dish two or three times daily. Except when sick, the animals took the milk very readily so that all was consumed within an hour, and thus little opportunity was given for much contamination.

The animals were weighed daily before feeding. This procedure gave the least fluctuation in the curves of body weight from day to day. Autopsies were performed as soon after death as possible. In some cases when the animals seemed to be dying, they were chloroformed and autopsied at once. Special attention was directed to the condition of the mouth and of the alimentary canal as well as of the bones.

Rôle of Inanition in Experimental Scurvy.

An ever present condition in the advanced case of scurvy is the lack of appetite for food; and the question arises, How much is inanition responsible for the symptoms produced? Funk (1914)

regards this factor as excluded. Lewis and Karr (1916) have shown that the food intake diminishes as the disease advances; but they discard fasting as being a very important factor in the production of the disease, inasmuch as autopsy reveals nothing indicative of scurvy. Farnam arrived at somewhat the same conclusion, and our evidence also supports this view.

We removed a number of animals from a normal diet of cabbage, carrots, and oats, and allowed them distilled water only. These animals declined rapidly and died in from 6 to 8 days after losing about 40 per cent in body weight. During this period they exhibited none of the usual symptoms of scurvy, such as swollen joints and tenderness. At autopsy, the bones were normal in appearance. There was no subperiosteal hemorrhage or fragility of the bones. The alimentary canal was empty except for a small amount of fluid in the stomach. The latter contained a few hemorrhages, while the intestines, including the cecum, were somewhat congested. The viscera were otherwise normal in appearance.

We have also observed, when producing scurvy on a variety of diets, that the swollen joints and tenderness often appeared while the animals were gaining in weight and still eating food with apparent relish. It seems therefore that inanition can only play a minor rôle, if any, in the production of the common symptoms of scurvy. It will be noticed in this connection that with scorbutic animals on cereal or soy bean diets, the curves of the body weights take a sudden drop about 10 days prior to death, and in this final period, the curves are similar to those seen in fasting. From this we may perhaps reasonably conclude that inanition is a result of the symptoms rather than a forerunner of them.

Scurvy-Producing Diets.

It is not intended to discuss here exhaustively all the diets that have been reported to have produced the scurvy of guinea pigs. We shall only mention those that have some direct bearing on our own experiments.

Cereals.—The classical method of producing scurvy in the guinea pig has been through the exclusive feeding of cereal grains. Theobald Smith used bran and oats; and Holst and Frölich mention a variety such as oats, rye, barley, maize, and oat-bran.

We had in different series a total of twenty animals on an exclusive oat diet. They all succumbed to scurvy and died on an average in 24 days. The course of the disease was the usual one. The symptoms appeared on about the 15th day after dieting was begun. The feces changed in color progressively from light brown to dark brown and then to almost black. The texture of the feces also changed from firm, well formed pellets to pasty masses which later became fluid in consistency. Several days before death blood was usually prominent in these discharges.

At autopsy the animals presented the anatomical picture of scurvy already described. The cecums of nine animals were congested; two showed impaction of feces as well as congestion; of the remainder, seven were negative and the condition of two was not recorded. The results are given in Table I.

Oatmeal.—Two animals on an exclusive oatmeal diet began to decline in weight at once, though the meal was eaten to some extent. The scanty feces were black and pasty. On the 7th day one animal died showing early symptoms of scurvy; the other was then given calcium lactate and sodium chloride *ad libitum*, and 1 gm. each of butter and dried brewers' yeast as a daily supplement to the oatmeal. This animal gained weight at once and recovered in 10 days almost all it had lost; but during this recovery it developed all the striking symptoms of scurvy. An intussusception caused death on the 22nd day of the experiment. Autopsy revealed the classical picture of scurvy. In addition, there was in both these animals impaction of feces as well as congestion of the cecum (see Table I).

Oats and Yeast.—Two animals were fed on oats and 1 gm. of dried brewers' yeast daily, the latter being added with the intent of furnishing the water-soluble vitamine known to be essential for nutritive well-being in the case of other species. The guinea pigs survived 12 and 15 days respectively, and exhibited only a comparatively small loss in weight. At no time during the course of the experiment were the usual symptoms of scurvy noted. Autopsy revealed in both animals a certain amount of hemorrhage in the walls of the alimentary canal, but the cecums appeared normal in this respect, and exhibited no impaction of feces. However, only one animal showed signs suggestive of scurvy in the bones. Although the results of this series are inconclusive, our

other experiments seem to indicate that yeast has no effect upon the production or symptoms of scurvy. For instance, in the preceding experiment with oatmeal, yeast was added among other things to the diet, but there seemed to be no effect upon the course of the disease. An addition of about 3 per cent dried yeast to the soy bean mixture described further on, also proved ineffective in preventing the disease.

Oats and Butter.—Two young animals were each given oats and 1 gm. of fresh butter daily; the latter being intended to supply sufficient fat-soluble vitamine. Though the butter was eaten greedily, the animals began to drop in weight at once, losing about 25 per cent in a week. On the 9th day one of them died, but the other was saved with fresh cabbage and carrots. At no time did these animals exhibit tenderness or swelling of the joints; but this is easily accounted for by the fact that nutritional failure came too rapidly for marked symptoms to become evident. Autopsy of the animal that died revealed subperiosteal hemorrhages near the knee joints. The congested cecum was impacted with feces. In some other diets, butter was fed as a supplement after scurvy had appeared, but there was no effect on the course of the disease (see Table I).

Oats, Calcium Lactate, and Sodium Chloride.—Three young animals were placed on this diet. The calcium salt was fed *ad libitum* in 5 per cent solution, while a crystal of rock salt provided the other ingredient. Two of the animals died in 14 and 15 days respectively, and though they showed no marked symptoms of scurvy during life, they presented the typical picture on autopsy. The third animal, though lighter in weight than the others, did not succumb until the 27th day. It began to show signs of tenderness and enlargement of the joints on the 7th day, the symptoms becoming more acute as time progressed.

These animals did not lose weight like those on an exclusive oat diet. In fact, they gained somewhat during the progress of the experiment; and at the end they were not very far below their initial weights. All showed congestion of the cecum, but only two had impacted feces at autopsy.

Apparently these inorganic salts improve the nutritional quality of the oats, but they do not affect the course or symptoms of scurvy in an appreciable manner. This fact is borne out by the

experiment with the oatmeal diet, in which the addition of the above inorganic salts along with yeast and butter failed to check the disease (see Table I).

Exclusive Barley Diet.—Two young animals were given an exclusive diet of seed barley. They ate this well, and maintained themselves without much loss in weight to the end of the experiment. On the 10th day, the whole barley grain was stopped and the ground, unsifted seed substituted, so that the animals could eat it more readily. On the 10th day, one animal showed first signs of scurvy, and succumbed by the 16th day. The other died on the 18th day, also showing slight symptoms. Autopsies confirmed the clinical diagnoses, though the hemorrhages around the bones were not very marked. Only one animal had an impaction of feces in the cecum (see Table I).

Soy Bean Flour.—It became increasingly evident to us that the customary method of producing scurvy in the guinea pig with the monotonous cereal diet is open to several points of criticism. The protein may be inadequate either qualitatively or quantitatively, or both. The inorganic salts are deficient, as we have indicated; and from the standpoint of McCollum the physical texture of the dietary may be unsatisfactory. Our work therefore led to a search for some better food material capable of dietary analysis.

The studies of Daniels and Nichols (1917) and of Osborne and Mendel (1917) upon the nutritive value of the soy bean suggested this material as a suitable basis for our experiments. The authors mentioned have demonstrated that the nitrogenous components of the soy bean are adequate for maintenance and growth, and that their utilization is good. Sodium, calcium, and chlorine are lacking. There is also present in the soy bean a liberal amount of the water-soluble vitamine and at least some of the fat-soluble vitamine.

In our early attempts to feed soy bean flour[2] we tried without success to induce our animals to eat it raw. Accordingly the flour was mixed with water into a paste which was dried at 70–80°C. This likewise was not readily eaten. Another modification of the diet proved only slightly more satisfactory. A mixture was made containing 100 gm. of raw soy bean flour, 4 gm. of sodium chloride, 4 gm. of calcium lactate, 4 gm. of dried brewers' yeast, and 125

[2] The soy bean flour was furnished by the Cereo Company, Tappan, N. Y.

TABLE I.

Some Scurvy-Producing Cereal Diets.

Guinea pig No.	Sex.	Weights. Initial.	Weights. Maximum.	Weights. Final.	Duration.	Diagnosis.	Remarks.
			Whole oat grains.				
		gm.	*gm.*	*gm.*	*days*		
1	♂	353	367	210	25	Scurvy.	
3	♂	486	506	317	30	"	Heated soy bean flour added on 24th day; eaten slightly. Died of pneumonia.
4	♂	342	344	204	24	"	
5	♀	330	354	181	26	"	
12	♂	357	367	244	20	"	
13	♀	286	296	172	22	"	
17	♀	365	365	189	22	"	Impacted cecum.
18	♀	354	370	221	23	"	
20	♀	308	322	194	22	"	
21	♀	201	228	136	23	"	Impacted cecum.
22	♀	372	412	240	24	"	
26	♀	333	339	233	24	"	
27	♀	375	375	200	23	"	
29	♂	357	361	211	24	"	
30	♂	472	510	315	23	"	
73	♀	460	485	267	30	"	Added 1 gm. dried cabbage on 25th day; not readily eaten.
74	♀	394	406	287	24	"	
75	♀	385	393	248	24	"	Added 50 cc. milk on 20th day.
76	♂	432	388	263	24	"	" " "
78	♂	427	435	258	27	"	Added 1 gm. dried cabbage on 24th day; partially consumed.
			Oatmeal.				
33	♀	245	245	198	22	Scurvy.	On 7th day added daily 1 gm. yeast, 1 gm. butter, and NaCl and Ca lactate *ad libitum*. Died of intussusception.
34	♂	201	201	127	7	"	

TABLE I—*Concluded.*

Guinea pig No.	Sex.	Weights.			Duration.	Diagnosis.	Remarks.
		Initial.	Maximum.	Final.			
Oats and dried yeast.							
		gm.	*gm.*	*gm.*	*days*		
35	♀	177	177	154	15	Scurvy(?).	
36	♂	173	173	145	12	?	
Oats and butter.							
37	♀	175	175	134	9	Scurvy.	
38	♀	190	190	139	8	"	Saved on 9th day with fresh cabbage and carrots.
Oats, calcium lactate, and sodium chloride.							
44	♀	148	188	130	15	Scurvy.	Impacted cecum.
45	♀	153	175	160	14	"	
46	♂	140	145	110	27	"	Impacted cecum.
Barley.							
39	♀	157	158	135	18	Scurvy.	Impacted cecum.
41	♂	153	155	139	16	"	

cc. of fresh, raw Jersey milk. These were made into a paste and dried on a radiator at 70–80°C. The animals ate this somewhat more readily than the previous mixture, though by no means freely. Upon this diet an animal barely maintained its weight. Symptoms of scurvy appeared early,—about the 8th day; and then followed great loss in weight which would have resulted in death in perhaps 6 more days had curative measures not been instituted. A supplement of 10 gm. of fresh cabbage daily was also scarcely enough to enable the maintenance of weight, though it prevented the appearance of scurvy. The stools were pasty and diarrheal from the very beginning of dieting, though no blood was present. Another noticeable feature was the marked diuresis in these animals.

It thus became evident that raw soy bean flour was not a satisfactory food for guinea pigs because they failed to eat it readily.

Osborne and Mendel have shown, in the case of the rat, that soy bean meal is not eaten readily when raw, but that cooking the food makes it more palatable. Certainly, cooking the flour or meal changes its flavor decidedly, giving it a taste similar to that of roasted peanuts; whereas the unheated material has the flavor of raw peas or beans. Our next series of experiments indicated a similar preference in the guinea pig, in that the cooked soy bean flour was consumed with eagerness.

In order to provide a uniform and reproducible procedure the soy bean flour was heated in an autoclave for 30 minutes at 15 pounds pressure (120°C.), this method being followed in all experiments where heated soy bean flour was fed. To correct the deficiency in inorganic constituents about 3 per cent each of sodium chloride and calcium lactate was added. We have no conclusive evidence as yet to indicate that heating under the conditions stated will leave unaltered the recognized essential dietary accessories (vitamines) in the soy bean flour. According to Chick and Hume the antiscorbutic vitamine, if such exists, is exceedingly sensitive to high temperatures. We therefore decided to add to the diet a liberal amount of water-soluble vitamine in the form of dried brewers' yeast (3 per cent), and of fat-soluble vitamine in the form of fresh, raw Jersey milk in quantity sufficient to add 5 per cent of butter fat. The milk also supplied additional water-soluble vitamine. These materials were mixed into a paste and then dried over a radiator at 70–80°C. for about 2 days until all the water was driven off.

This soy bean cracker was fed to two animals that ate it with great readiness. They gained in weight very rapidly,—better than on the usual carrots-cabbage-oats diet. At first their appearance was satisfactory. On about the 10th day, however, they exhibited a tenderness of the wrist and ankle joints, though they were still eating well and gaining in weight. Then the condition became more severe. The joints swelled to twice or three times the normal diameter, and spontaneous fracture of the wrist occurred in one animal. Appetite diminished, and a sharp nutritive decline ensued. Curative measures mentioned below were instituted.

A number of other animals were put on this diet and all exhibited the identical clinical symptoms described above. Marked

diuresis was present, a condition always occurring where the above salt supplement was added. The feces very early became of a pasty consistency and then almost fluid. Toward the end blood was sometimes present in the feces, though not very frequently.

Apparently the foregoing diet is satisfactory from the nutritive standpoint in every way except (1) the physical texture is poor in that roughage is lacking, and (2) an antiscorbutic property is absent. As will be seen later, we have attempted an analysis of this problem.

A feeding experiment with a ready made mixture of one-third soy bean flour, two-thirds whole corn-meal, and a little bone-ash also produced symptoms of scurvy, but in this case there was marked constipation (see Table II).

Contrary to the unfavorable effect of the soy bean diet on the nutrition of the guinea pig, we have found the same food mixture

TABLE II.

Scurvy-Producing Soy Bean Diets.

Guinea pig No.	Sex.	Weights. Initial.	Weights. Maximum.	Weights. Final.	Duration.	Diagnosis.	Remarks.
			Unheated soy bean flour.				
		gm.	*gm.*	*gm.*	*days*		
87	♀	315	315	224	24	Scurvy.	First symptoms in 9 days. For last 10 days received 20 per cent lactose with diet.
86	♀	286	286	259	—	Normal.	Received 10 gm. raw cabbage daily.
			Soy bean "cracker."				
55	♀	318	382	273	19	Scurvy.	Saved 19th day, with 10 gm. raw cabbage daily.
56	♀	263	340	288	20	"	Saved on 20th day with 3 gm. dried cabbage daily.
			Soy bean flour-whole corn meal-bone ash mixture.				
151	♂	120	136	114	13	?	Constipation marked.
153	♂	205	205	168	12	Scurvy.	" "
154	♂	303	304	224	19	"	" "
155	♂	276	276	173	7	"	" "

to be adequate for the rat. For example, six rats living on this diet for more than 100 days grew at a normal rate and developed no evident nutritive defects. Two of them were anesthetized and carefully autopsied, but they revealed none of the usual macroscopic signs of scurvy. This is what one would expect in the light of the experiences of Osborne and Mendel (1917). This difference in response of the guinea pig and rat to comparable diets has been pointed out by McCollum and Pitz and by Funk.

Milk[3] *in the Production of Scurvy.*

Jackson and Moore have shown quite conclusively that either raw or heated milk will produce scurvy in the guinea pig. They note that the younger animals succumb most readily while older ones will continue for quite a while before exhibiting noticeable symptoms. McCollum and Pitz have used fresh milk and oats as the basis for their experiments, but apparently fail to recognize the difference in susceptibility in animals of different age. They ascribe some of their divergent results to an inherent unsuitability of the guinea pig to nutritional experimentation. However, neither Jackson and Moore nor McCollum and Pitz give any record of the daily intake of milk for each animal. They merely state that the milk was fed *ad libitum*, hence there is no means of knowing the intake.

The significance of this becomes immediately apparent in the light of the recent work of Chick, Hume, and Skelton (1918), who found that a *large* daily ration of milk was required to prevent the onset of scurvy in the guinea pig. Indeed, according to them, a diet almost exclusively of milk became necessary. Short of this they were unable to prevent scurvy, although the onset of symptoms was delayed and growth of the animal maintained in proportion to the quantity of milk consumed daily. They found, for example, that when 30 to 50 cc. of raw milk were taken daily, death from scurvy ensued in about 30 days. When 50 cc. were consumed daily, the life of the animal was prolonged to 75 days. When the daily intake of milk ranged from 85 to 135 cc., good health was maintained throughout the 3 months of their experi-

[3] Cow's milk only is referred to in these statements. In our experiments fresh, raw Jersey milk of "certified" grade was nearly always used.

ment. These results are remarkably consistent and help to explain the results we have obtained in feeding milk.[4]

Four half-grown animals were started on a diet of oats and 35 cc. of milk daily. The amount of oats eaten varied considerably. On some days 20 to 30 gm. were ingested per animal; on others, the consumption fell to about 5 gm.; however, in the main, the oats were consumed in considerable quantity until the daily amount of milk was greatly increased. At first, water was kept from the animals lest its use might tend to reduce the consumption of milk, but later this was found not to be the case. On the contrary, the animals at all times drank the milk in preference to water. Accordingly, distilled water was always made available freely. These animals gained weight somewhat or maintained themselves for about 25 or 30 days, whereupon they began to lose very slightly. The oldest animal broke a leg in a fall of 2 feet from a shelf to the floor. It was also observed that all of them failed to show the usual alertness. However, tenderness of the joints was not yet observed. On the 29th day, the daily amount of milk was increased to 40 cc. per animal; on the 34th day, to 50 cc. On the 36th day all the animals exhibited tenderness of the joints, while two (the heaviest and the lightest) had distinct enlargements of the knee joints. On the 40th day, the daily milk ration was increased to 60 cc., and on the 43rd day to 70 cc. This was continued for 15 days, during which two animals gained in weight, one maintained itself, and the fourth died. Then after the 58th day, two of the animals were given 100 cc. of milk daily, while the third was continued on 70 cc. with a daily supplement of 1 gm. of dried yeast. The animals receiving 100 cc. of milk soon began to drop in weight. After 3 days they stopped taking more than about 35 to 65 cc. The cecums could be palpated and indicated that there was impaction of the feces. On the 64th day death ensued. The animal on 70 cc. of milk plus 1 gm. of dried yeast daily survived until the 91st day.

Two younger animals started on the oat and milk diet when weighing about 175 gm. Following an initial intake of 15 cc. daily, the amount was increased on the 16th day to 30 cc.; on the

[4] Funk likewise states that large quantities of milk are needed to prevent scurvy.

TABLE III.

Milk and Oats.

Guinea pig No.	Sex.	Weights.			Duration	Diagnosis.	Remarks.
		Initial.	Maximum	Final.			
		gm.	*gm.*	*gm.*	*days*		
8	♀	305	350	270	56	Not scurvy.	Milk intake of each 35 cc. daily. Increased to 40 cc. on 29th day; to 50 cc. on 34th day. Symptoms appeared on 36th day. Milk increased to 60 cc. on 40th day; to 70 cc. on 43d day; and on 58th day, Nos. 9 and 10 received 100 cc. daily. Autopsies revealed marked impaction of feces.
9	♀	330	388	300	64	" "	
10	♂	325	368	260	64	" "	
11	♀	400	426	278	91	Scurvy(?).	Received on 58th day addition of 1 gm. dried yeast also.
31	♀	176	273	233	77	Scurvy(?).	Milk increased progressively from 15 to 80 cc. daily on 65th day. Discontinued on 77th day. (See Table V.)
32	♀	174	274	198	57	Not scurvy.	Milk increased progressively from 15 to 70 cc. daily on 31st day. Contracted pneumonia on 52d day.
92	♂	123	153	123	26	Scurvy.	First symptoms on 11th day. On 21st day added mixture of 20 per cent lactose and 80 per cent oatmeal.
93	♀	268	300	170	27	"	First symptoms on 15th day. On 21st day added mixture of 20 per cent lactose and 80 per cent oatmeal.

21st day, to 40 cc.; on the 27th day, to 50 cc.; and on the 31st day, to 70 cc. Painful joints were in evidence on the 23d day while the animals were still gaining in weight. The abdomens became

distended and the coats less sleek. On the 57th day an animal succumbed to pneumonia. The other continued on this diet to the 76th day as reported below, when curative measures were attempted with dried cabbage.

Autopsies of the five animals that died on the oat and milk diet revealed only slight lesions of scurvy in one of them. Four showed no subperiosteal hemorrhages or fragility of the bones. A questionable enlargement of the joints was found in two cases. The fifth (the animal that had broken its leg) did show marked enlargement of the joints and great fragility of the bones. In none of these animals was there any sign of hemorrhage, subcutaneous, intramuscular, or subperiosteal. All of them exhibited most decided impaction of feces in the cecum which was distended with chalk-colored, hard, dry material (see Table III).

With these facts before us, our diagnosis on autopsy was as follows: In three cases there was constipation without scurvy; in one case death was due to pneumonia, constipation being present and scurvy absent; and in one case constipation accompanied scurvy. These conclusions are based solely on macroscopic examination of the tissues.

Milk forms a singularly constipating diet, especially for the guinea pig; and it seemed apparent that two of the animals died because the milk intake was increased from 70 to 100 cc. daily. Observations on the other animals also showed the constipating character of the diet. It is natural to assume that if constipation is the predisposing or causative agent of scurvy, a prolonged period on a constipating diet will produce more severe symptoms of the disease. Yet our experiments reveal a different outcome apparently. By the end of the first 25 days of dieting, while the animals were receiving a comparatively small amount of milk, they developed noticeable clinical symptoms of scurvy. Then, as the amount of milk was progressively increased the symptoms receded in intensity, so that at autopsy the usual picture of scurvy was practically missing.

These results fit in very closely with the findings of Chick, Hume, and Skelton. It should, of course, be remembered that their experiments were preventive in nature, while those here reported might be described as curative.

Relation of Roughage to the Production of Scurvy.

McCollum and Pitz have repeatedly stressed the argument that scurvy is due to unsatisfactory physical factors in the diet and to injury wrought through the agency of microorganisms inhabiting the alimentary canal,—not to the lack of an antiscorbutic vitamine. They have presented experimental evidence claimed to all but prove their point.

As a scurvy-producing diet, they fed oats and fresh raw milk *ad libitum*, and directed their attack upon the problem of hastening the elimination of the feces. We have already indicated from the work of Chick, Hume, and Skelton, and from our own experiments, that milk, unless consumed in limited quantites, may be the opposite to a scurvy-producing diet.

If physical factors of the diet are primarily responsible for the production of scurvy of the guinea pig, as the hypothesis of McCollum and Pitz would postulate, then suitable changes in the quality and quantity of roughage may be expected to produce somewhat corresponding changes in the course and symptoms of scurvy. With this point in view, we have tried a number of diets in which the roughage factor was varied within the experimental limitations. Obviously, fresh succulent vegetables such as have been proposed cannot be used to solve the problem, for they may contain accessory substances (vitamines, etc.) beside the bulk-giving cellulose. Therefore a number of inert, indigestible materials were incorporated in scurvy-producing diets in order to study this single roughage factor.

Oats and Sawdust.—It has already been amply shown that a diet of oats alone will produce scurvy in a short time. The following was an attempt to repeat Farnam's work under closely comparable conditions. The animals were bedded on clean sawdust which was changed three times weekly. Three pigs on this diet died in 15, 20, and 21 days respectively. They all ate more or less of the sawdust as could be seen by the feces, though the exact amount consumed was not measured. Autopsy also showed this material in the alimentary canal. Only one animal had a slight impaction of feces in the cecum, whereas the others had fluid feces. The usual findings of scurvy were present in all the animals. The time of onset of the disease or death was not

appreciably different from what is found on an exclusive diet of oat grains (see Table IV).

Oats and Filter Paper.—Three animals were placed on an oat diet, and shredded filter paper was supplied *ad libitum*. Early in the experiment, about 3 gm. of paper were consumed per guinea pig per day. This continued until about 3 days before symptoms of scurvy became evident, when no more paper was eaten. The animals died in about 20 days, all with signs of scurvy. The usual picture was found at autopsy. Two of the animals had impaction of feces in the cecum; in the third, the feces were fluid (Table IV).

Oats and Hay.—Two animals on this diet first showed symptoms on the 15th day and succumbed about the 21st day. Their curves of body weight were similar to those of animals on an exclusive diet of oat grains. The feces did not change in consistency. The pellets were well formed though blackish instead of light brown. Autopsy showed the usual picture of scurvy, with no impaction of feces in the cecum (Table IV).

Oats, Hay, and Cod Liver Oil.—Three animals were placed on this diet. The hay was provided as bedding, and 1 cc. of cod liver oil was given daily. The curves of body weight were practically the same as those of animals on oats alone or oats and hay. Two succumbed on the 24th day and one on the 26th day of dieting. The symptoms of scurvy appeared about the 18th day. The usual picture was seen at autopsy, except that congestion of the viscera was marked. The cecums had some hemorrhagic patches and contained a little fluid feces. The "fat constipation," noted by McCollum and Pitz in comparable diets, was not evident in these animals. The use of cod liver oil was suggested by the experiments of Hess and Unger (1918); and the appearance of scurvy on this diet suggests that rickets is probably not a complicating factor in view of the reported specific action of cod liver oil upon rickets (see Table IV).

Soy Bean "Cracker" plus Cellulose.—Even after being supplemented by inorganic elements, and addition to its content of fat-soluble and water-soluble vitamines, soy bean flour whether heated or raw failed to prevent scurvy. It next became necessary to learn whether alteration of the physical texture of this diet would change its scurvy-producing property.

TABLE IV.

Scurvy-Producing Diets Containing Roughage.

Guinea pig No.	Sex.	Weights.			Duration.	Diagnosis.	Remarks.
		Initial.	Maximum.	Final.			
Oats and sawdust.							
		gm.	*gm.*	*gm.*	*days*		
51	♀	319	323	198	20	Scurvy.	
58	♀	353	353	222	21	"	Impacted cecum.
60	♀	364	364	222	15	"	
Oats and filter paper.							
2	♂	410	420	261	21	Scurvy.	Refused to eat paper after 10th day.
6	♀	268	286	182	21	"	" " "
7	♀	328	330	197	19	"	" " "
Oats and hay.							
15	♀	180	195	126	21	Scurvy.	
16	♂	172	180	115	20	"	
Oats, hay, and cod liver oil.							
52	♂	267	293	178	24	Scurvy.	
57	♀	354	362	206	26	"	
59	♀	510	516	220	24	"	
Soy bean cracker plus cellulose.							
79	♀	257	323	199	25	Scurvy.	6 per cent cellulose.
80	♂	224	302	198	18	"	6 " " "
89	♂	369	580	545	86	Normal.	6 " " " Received 10 gm. raw cabbage daily.
84	♀	487	520	290	57	Scurvy.	18 per cent cellulose. Added 20 per cent lactose on 15th day for 7 days. Decline arrested with 10 gm. raw cabbage daily on 22d day. Discontinued cabbage on 33rd day.
85	♀	307	345	223	21	Scurvy.	18 per cent cellulose. Added 20 per cent lactose daily on 16th day.
90	♀	262	280	195	18	"	" " "

Thoroughly pulped filter paper was incorporated into the soy bean "cracker," to the extent of 7.2 per cent, and butter fat, 4.3 per cent. This "cracker" was very light, and eaten readily by the animals. Another lot was made containing 17.9 per cent cellulose in the form of paper pulp and 6 per cent butter fat. These were fed to different groups of animals with uniform results. The symptoms of scurvy appeared in about 10 days, and death was certain to supervene a short time later unless curative measures were instituted. Impaction of the feces was uncommon in the animals on these diets, though injury to the cecum as evidenced by hemorrhage or congestion was often present.

From the character of the feces in these series, it may be said that for a while at least (in the first three-quarters of the dieting period), the feces more closely resembled the normal in consistency. This would lead one to conclude that perhaps during this period evacuation of the bowels was more readily accomplished than on some of the other diets mentioned. If this is true, then ill effects due to retention of feces must also have been minimized to some extent. Yet in spite of this favorable circumstance, there was no appreciable effect upon the disease. Apparently, the onset of scurvy does *not* depend upon a previous condition of constipation.

A consideration of all the facts we can bring together makes it increasingly evident that the simultaneous exhibition of constipation and a certain symptom complex with a given diet are no *prima facie* evidence that the constipation is responsible for the appearance of the symptom complex. It may well be that these are merely intercurrent conditions. It is easily conceivable that constipation, through its complication of the symptoms, may enhance the severity of the disease (cf. Gerstenberger, 1918).

Antiscorbutic Diets.

Orange Juice.—This represents the classic agent for curing scurvy. A daily dose of 5 cc. added to a scurvy-producing diet will preserve the animal in good health indefinitely. When given as a curative treatment to scorbutic animals, we have found orange juice entirely effective in initiating prompt improvement and ultimate cure. For the latter result it is necessary to begin treat-

ment reasonably soon, and not to wait until a short time before death, when almost irreparable damage has been done to the organism. Orange pulp seems to be equally effective as an antiscorbutic (Table V).

Fresh Cabbage.—This is also considered a very effective antiscorbutic agent. We have kept animals for at least 3 months on an exclusive diet of cabbage and oats. One female, fed oats and about 35 gm. of cabbage daily, reared a litter of three young. Animals have also been maintained in splendid condition on soy bean cracker plus 10 gm. of fresh cabbage daily for more than 90 days. Fresh cabbage will also act as a curative agent if administered early enough (Table V). Our experiments indicate that fresh carrots have similar properties.

Dried Cabbage.—This product was prepared by spreading fresh cabbage leaves over a radiator at 70–80°C. for 2 days or more. About 90 per cent in weight was lost as moisture. This cabbage was first tested on an animal that was receiving 80 cc. of milk daily plus oats *ad libitum* (comparatively little of the oats being consumed). It had been living for 76 days on an increasing milk diet plus oats. For 15 days previously it failed to take much of the milk fed, and was beginning to decline. Then 1 gm. of dried cabbage and 80 cc. of milk were fed daily. The cabbage was consumed greedily as soon as fed, and there resulted a prompt and continuous increase in weight. Within several days no milk remained unconsumed. 15 days later, the amount of dried cabbage was increased to 3 gm. daily and was continued for 10 days. There was, however, no increase in rate of gain over that secured with 1 gm. Accordingly, the 3 gm. ration was discontinued in favor of the 1 gm. This animal has been on 1 gm. of dried cabbage plus 80 cc. of milk for more than 110 days and has increased in weight from 233 to 590 gm.

In another case we tried the curative effect of dried cabbage after an animal had developed scurvy on soy bean cracker. The animal had lost a little over 50 gm. from its maximum, but was still well above its initial weight. It was therefore little damaged by the disease as yet. This animal received 3 gm. of dried cabbage daily in addition to the soy bean cracker and immediately began to pick up in weight. In 16 days it had gained 121 gm. and symptoms of scurvy had disappeared. The dried cab-

bage was then discontinued, and the animal maintained itself with slight increase in weight for 2 weeks longer. At the end of this period it began to decline very rapidly to death.

These examples indicate that the dried cabbage may have acted in a larger capacity than merely as a type of roughage. In the first case, 1 gm. of dried cabbage represented only a small portion of the total daily intake of food. It is estimated that in addition

TABLE V.

Antiscorbutic Diets.

Guinea pig No.	Sex.	Weights. Initial.	Weights. Maximum.	Weights. Final.	Duration.	Diagnosis.	Remarks.
		gm.	*gm.*	*gm.*	*days*		
Orange juice or pulp.							
47	♂	258	444	432	113	Scurvy cured.	Oat diet; scurvy 12th day and added 30 cc. orange juice. On 35th day changed to 5 cc. orange juice plus soy bean cracker. Still living, normal.
77	♂	415	428	325	43	" "	Oat diet; scurvy 17th day and added 5 cc. orange juice. Began recovery promptly. Chloroformed on 43d day, showing receding lesions of scurvy
54	♀	406	425	408	45	" "	Oat diet; scurvy 19th day and added pulp of ½ orange daily. Began recovery at once. Chloroformed on 45th day, showing receding lesions of scurvy.
Raw cabbage.							
23	♀	364	830	700	150	Normal.	Gave birth to litter of 3 which were successfully raised on same diet. This animal is representative of 15 others on diet of oats and raw cabbage.

TABLE V—*Concluded.*

Guinea pig No.	Sex.	Weights. Initial.	Weights. Maximum.	Weights. Final.	Duration.	Diagnosis.	Remarks.
			Dried cabbage.				
		gm.	*gm.*	*gm.*	*days*		
31	♀	233	626	590	120	Normal.	(See Table III.) On oats and 80 cc. milk daily. Added 1 gm. dried cabbage for first 15 days, and 3 gm. from 15th to 25th day. From 25th day resumed supplement of 1 gm. dried cabbage daily.
61	♀	258	307	247	82	Not scorbutic.	Refused to eat last 6 days. Dried cabbage, 3 gm., and oats.
62	♀	245	245	151	50	" "	Died of pneumonia. Dried cabbage, 3 gm., and oats.
70	♂	330	478	477	102	Normal.	Chloroformed; no scorbutic lesions. Dried cabbage, 3 gm., and oats.
72	♀	384	494	478	103	"	Dried cabbage, 3 gm., and oats.
63	♀	242	445	420	125	"	Dried cabbage, 3 gm. and oats. On 68th day change to soy bean cracker plus 1 gm. dried cabbage daily.
			Germinated cereal grains.				
64	♀	295	349	207	70	Not scorbutic.	On germinated oats.
65	♀	355	356	283	72	" "	" " "
66	♀	325	357	242	47	" "	" " "
67	♂	395	442	298	53	" "	" " barley.
68	♀	352	352	267	56	" "	" " "
69	♂	345	350	239	19	" "	" " " Died of pneumonia.

to 80 cc. of milk, the animal consumed at least 10 gm. of oats when nutrition was restored. Even the 3 gm. of dried cabbage represented a small part of the total intake, and the ability of the

animal to maintain itself for 2 weeks after removal of the dried cabbage is of significance.

Five animals placed on a diet of oats and dried cabbage (about 3 gm. per guinea pig per day) continued for long periods without signs of scurvy. One died of pneumonia on the 50th day, and another apparently of inanition on the 80th day. No macroscopic lesions of scurvy were found at autopsy. The other animals survived over 100 days without any signs of scurvy (see Table V).

Germinated Oats and Barley.—Three guinea pigs were placed on a diet of sprouted oats, and three others on a diet of sprouted seed barley. The animals did not make any considerable gains in weight but maintained themselves for a comparatively long period. No signs of scurvy were found at autopsy (see Table V). This result confirms the report of Fürst (1912), who found that germinated oats prevented the outbreak of scurvy in guinea pigs, and of Weill, Cluzet, and Mouriquand (1917), who found that barley in the 3rd day of germination is a better complete food for guinea pigs than is the ungerminated grain. Chick and Hume (1917) also report that germinated cereals and pulses possess marked antiscorbutic properties.

Alleged Antiscorbutic Property of Lactose.

Pitz (1918) has reported that certain carbohydrates apparently exert a curative or delaying action upon the course of scurvy in the guinea pig. Lactose added in sufficient quantity to a milk diet seemed to act as an antiscorbutic, more effectively than did dextrin or sucrose. However, acceptance of his observation as a fact must be regarded with caution until certain essential points have been cleared up.

The fact that the quantity of milk ingested by each animal was not controlled suggests, as an equally plausible explanation, that more milk may have been consumed by those guinea pigs exhibiting recovery from scurvy on the diets of carbohydrate plus milk, in the experiments of Pitz. Moreover, the degree of purity of the lactose was not stated and is another factor requiring consideration. Small unsuspected amounts of vitamine are known to have been carried along by the lactose used in certain nutrition experiments with isolated foodstuffs (Drummond, 1916).

Two young animals were placed on a diet of 30 cc. of milk daily plus oats *ad libitum*. They developed symptoms of scurvy in 11 and 15 days respectively. On the 21st day they were changed to a diet consisting of 20 per cent lactose[5] and 80 per cent oatmeal plus 30 cc. of milk daily. This did not seem to affect the course of the disease and both animals were dead on the 27th day (see Table III, Nos. 92, 93). It may be, however, that curative measures were not begun soon enough in these cases.

Seven animals that had contracted scurvy on the soy bean cracker diet were given 20 per cent lactose in addition to this diet, without any appreciable effect upon the course of the disease being noted (see Table IV). One guinea pig given a prophylactic treatment of 20 per cent lactose added to the soy bean diet, died in 23 days. There was no opportunity to study the effect of a less pure lactose, and we therefore do not know whether the suspected impurity of the lactose may be responsible for the antiscorbutic action such as has been reported by Pitz. The results here reported do not confirm his findings.

DISCUSSION.

It is clear from our experiments that the so called water-soluble and fat-soluble vitamines, so significant in the nutrition of certain species, are not the primary factors concerned in the scurvy of the guinea pig. Moreover, while an addition of inorganic salts improves the nutritive quality of such scurvy-producing diets as cereal grains, it has no effect upon the disease. This is in agreement with the findings of McCollum and Pitz and of Chick and Hume.

A food mixture like our soy bean cracker, which presumably contained all the known essentials of a perfect diet, failed to prevent scurvy in the guinea pig. Yet addition of small amounts of orange juice, raw cabbage, or dried cabbage prevented or cured the disease. Like some other diets, the soy bean cracker was able to maintain *rats* in apparent good health for a long period while guinea pigs failed upon it. This shows an apparent difference in the food requirements of the two species. Whether this dif-

[5] The lactose of the highest purity obtainable was prepared by Dr. C. S. Hudson, of the Bureau of Chemistry, Washington, D. C.

ference is merely qualitative or quantitative is as yet difficult to decide. It may also be that the manifestations of scurvy in the rat are more obscure and develop more slowly than in the guinea pig.

It has been found in the foregoing experiments that *dried* cabbage apparently exerts some antiscorbutic effect. This result is contrary to the findings of Holst and Frölich and of Chick and Hume, who reported the failure of *dried* vegetables to prevent or cure scurvy. It appears that there is here a factor at play which has not been wholly under control.[6]

It is highly improbable that constipation is the predisposing cause of scurvy, as can be seen from our results in feeding milk. A small amount ingested daily permitted the onset of symptoms of scurvy, while larger quantities, which were not less constipating, caused the scorbutic symptoms to disappear. Moreover, aids to the ready elimination of feces, like differing amounts and kinds of roughage, caused no corresponding change in the course of the disease. Contrary to the findings of McCollum and Pitz, Hess (1918) was unable to protect or cure animals with daily additions of mineral oil to a scurvy-producing diet. Neither was he able to induce active scurvy in a latent case by the production of marked constipation with morphine. Recently, this author[7] reported the cure of scurvy by means of intravenous injections of orange juice. This successful antiscorbutic effect, produced by *parenteral* treatment, apparently was not due to specific relief from constipation or its attendant intestinal complications.

On the other hand, one must not forget that marked scorbutic symptoms have been induced in guinea pigs on laxative diets. Infantile scurvy has also been found to occur quite as often in connection with diarrhea as with constipation (cf. American Pediatric Society Committee, 1898). It seems clear, therefore, from the foregoing facts that constipation is not the causative factor in scurvy as McCollum and Pitz would have us believe. It must be remembered that the vital resistance of a scorbutic animal is undoubtedly lowered, thus rendering it more susceptible to unfavorable conditions like infections and toxemias.

[6] Cf. Givens, M. H., and Cohen, B., *J. Biol. Chem.*, 1918, xxxvi, in press.

[7] Hess, A. F., *Proc. Soc. Exp. Biol. and Med.*, May 15, 1918.

The suggestion of Pitz that lactose acts as an antiscorbutic through its influence on the intestinal flora cannot be accepted without more convincing evidence. We know that the ingestion of comparatively large amounts will change the intestinal flora to an acidophilic type; and it is reasonable to expect a corresponding alteration of intestinal conditions. This, however, cannot be termed an antiscorbutic effect. The experiments reported by us do not reveal any such quality in highly purified lactose fed in carefully controlled diets.

SUMMARY.

Experimental scurvy of the guinea pig is demonstrable at will with suitably chosen diets.

Exclusive diets of cereal grains like oats and barley produce the disease. Germinated oats or barley prevent the appearance of scurvy even when fed for comparatively long periods.

Scurvy arises on a diet of soy bean flour, even when the latter is supplemented with fat-soluble and water-soluble vitamines, inorganic salts, and cellulose.

Small additions of raw milk do not prevent the onset of scurvy. Larger quantities cause the symptoms to disappear.

Roughage in the diet plays, if anything, a minor accessory rôle in the prevention of scurvy. This disease is not essentially dependent upon constipation as a causative factor though the latter may aggravate the symptoms.

Cabbage seems to retain some antiscorbutic properties even when dried.

Contrary to certain current statements, highly purified lactose, fed in conjunction with a scurvy-producing diet, does not appear to have any effect upon the course of the disease.

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ADDENDUM.

Since this paper was sent to press the following papers of interest in relation to our experiments have appeared:

Rappleye, W. C., A note on experimental scurvy in the guinea pig, *Boston Med. and Surg. J.*, 1918, clxxix, 98.

Chick, H., Hume, E. M., and Skelton, R. F., The antiscorbutic value of cow's milk, *Biochem. J.*, 1918, xii, 131.

Harden, A., and Zilva, S. S., The differential behaviour of the antineuritic and antiscorbutic factors towards adsorbents, *Biochem. J.*, 1918, xii, 93.

These contributions contain numerous items in accord with our experience and our interpretations.

ON INDICATORS IN ANIMAL TISSUES.

By W. J. CROZIER.

(*Contributions from the Bermuda Biological Station for Research, No. 93.*)

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1. With the hope of discovering in the tissues of marine animals intracellular pigments capable of behaving as indicators of acidity, and having in mind also the possible significance of such materials for the general theory of animal pigmentation in its more purely biological aspects, I have from time to time made examination of coloring matters extractable from various pigmented animals. The occurrence of substances of this kind I find to be relatively frequent. A few of them seem adequately situated within the organism, and of an appropriate delicacy, so far as their color changes are concerned, to be capable of affording some indication of the normal intracellular acidity. By far the most satisfactory of these substances, however, is the one which I have already studied in *Chromodoris zebra* (Crozier, 1916*a*).

Table I gives in outline some of the more obvious characteristics of a number of pigment substances, from various marine invertebrates, which exhibit reversible acid-alkali color changes. The acidities at the points of color transition were determined in several ways; most usually, by adding a small volume of the concentrated pigment extract to "buffer" mixtures of known acidity, in other cases by determining the hydrogen ion concentration in solutions in which the respective substances appeared to be "neutral."[1] It is, of course, necessary to employ freshly prepared extracts; most tissue pigments are bleached by sunlight and air, or changed in other ways on standing in solution. Except in one case (*Chromodoris zebra*), no effort was made to determine the per-

[1] These observations were made with indicators and gas-cell apparatus obtained, for use mainly in the study of another problem, through a grant from the C. M. Warren fund of the American Academy of Arts and Sciences to the Director of the Station.

TABLE I.

Color Changes of Indicators from Some Animal Tissues.

Animal from which obtained.	Acidity, pH.									
	4.0	5.0	5.6	6.0	7.0	7.5	8.0	8.5	9.0	10.0
Sponge A.....		Yellow → brown →			violet		→ deep purple			→ blue.
Sponge B.... (i).						Scarlet →	scarlet, brown dichromaticity	→	brown, muddy yellow.	
Sponge B.... (ii).	Colorless →		green.							
Velella velella (L.).								Colorless →	yellow →	orange.
Mellita..... (i).						Colorless →	green.			
Mellita..... (ii).	Orange → orange-red,			purple	——	——	——	——→ blue.		
Stichopus.....	Orange → reddish						——→ purple.			

The heavy vertical line signifies region of sharpest color change.

centage transformation of the indicator required for a visible "end-point." In this one instance the percentage transformation was 23 to 27 per cent,—about at the limit of usability for "good indicators" in ordinary titrations.

These notes supplement a previous brief article on indicators in animal tissues (Crozier, 1916*b*), and are now published because the investigation is not to be continued for the present.

1. *Sponge A.*—A brilliantly yellow sponge, found usually in shore caves. The surface of the sponge substance is thrown into sharp prominent conules, connected with the body by thin ridges. The outermost region of these thin projections is frequently purple; fine purple streaks occur on the body surface generally. The yellowest individuals are found in the darkest situations; in more illuminated locations the intensity and extent of purple coloration is greater. They are sometimes (rarely) found, freely exposed to the sun, upon the carapace of a "decorator crab" (*Mithrax*); in this case, the whole sponge is usually deep lavender or purple in color. The pigment is soluble in water, is made bright yellow by acids, and blue to purple by alkalies. These colors are very like those of alizarin-sulfonate solutions. After death, the substance of the sponge becomes dark purple. Alkaline solutions precipitate the substance in purple form. The yellow solutions exhibit a general absorption in the blue region of the spectrum, with, possibly, a weak band in the blue-green.

2. *Sponge B.*—(i) Brownish in external appearance, growing attached to the lower portions of *Halimeda* and other plants in a mangrove "creek." The inner substance of the sponge is a deep scarlet color, the thin outer coating deep brown. The scarlet pigment, extracted with water, gives a clear crimson solution with decided brown dichromaticity. Alkalies make this extract brownish yellow or orange; acids, violet. These color changes are very vivid, instantly effected by weak NH_4OH or by butyric acid, and freely reversible. Acid solutions slowly precipitate the pigment in purplish flocculent masses. Neutral solutions when acidified, lose their brown dichromaticity, which, however, reappears on neutralization. The absorption spectrum does not differ materially in acid, neutral, or alkaline solutions. In thin layers, two well defined bands are shown, one in the yellow-green and another in the green-blue; the former is the more pronounced, and is resolvable, in dilute solutions, into a minor band in the yellow and a major one in the yellow-green.

The pigment may be "purified" by repeated precipitation with acid and re-solution in weak alkali. In dilute solution the color change of this "purified" pigment is very sharp. Its behavior strongly suggests that the color of this substance is largely determined by its state of aggregation.

(ii) By the action of concentrated NH_4OH for 24 hours, the purple precipitate filtered from an acid solution was first made deep brown (like the outside of the living animal), and subsequently dissolved to a green solu-

tion, which was decolorized (but not made scarlet) by acid. The change, green $\rightleftarrows$ colorless, was reversible, but the material was quickly destroyed and precipitated in acid solution. The green solution gave a pronounced absorption band in the red-orange.

Sponges A and B were unidentified members of the *Aplysinidæ*, in one genus of which Krukenberg long ago found a material apparently not unlike that described for A. These substances have also considerable superficial resemblance to the "spongioporphyrin" of Lankester and MacMunn. The descriptions of various *Aplysinas* in taxonomic works (based on preserved specimens) seem to show that similar pigment materials are quite generally distributed among the representatives of this family.[2] One gains the impression that possibly (stereochemically ?) differences exist in the pigments of different allied species analogous to the specific differentiation of tissue proteins.

3. Velella velella (L.).—The blue pigment of this colonial hydrozoan gives an orange extract with 70 per cent alcohol. It is made bright orange by alkalies, decolorized by acid.

4. Stichopus moebii Semp.—The most anterior portion of the esophagus of this large holothurian contains "echinochrome," which is normally of a reddish purple hue. Alkalies intensify the color, and with acids it becomes bright orange. The pigment cannot satisfactorily be extracted. This "echinochrome" is apparently not so well situated for penetration studies as in the holothurian studied by Harvey (1914).

5. Mellita sexiesperforata Leske.—(i) For a note on the alkali-greening substance in the tissues of this sand-dollar, cf. Crozier, 1918*a*.

(ii) The ovaries of the female *Mellita* contain an "echinochrome" pigment, of which aqueous extracts are easily prepared. It exhibits the color changes characteristic of the "echinochrome"—"antedonin" pigments obtained from numerous other echinoderms.

2. It is instructive to compare certain of these findings with the colors of the living tissues concerned. In making these comparisons, care must be exercised that the color of a pigment within a tissue, and the variations of this color, are not due to conditions of the pigment determined otherwise than by the immediate acidity. We cannot be entirely certain that in any given case the indicator is sufficiently "free" to permit of its ideal performance, but the general concordance among the suggestions which the different instances afford, with reference to the reaction of the intracellular fluids, is favorable to the idea that these suggestions are of real value. From the data embodied in Table II, it will be

[2] Zoological texts refer to Krukenberg's pigment from an *Aplysina* as "remarkable," apparently under the impression that it is unique.

seen that all the cases in which there is reason to regard the natural condition of the pigment as at all comparable with its state when extracted, apparently refer to an intracellular acidity of between pH = 6.0 and pH = 7.6. The tissues of marine animals are in general more acid than the surrounding sea water.

The pigmentation of the two sponges studied might suggest that the more superficial tissues, especially when exposed to light, become so permeable that their contents more nearly approach the alkalinity of sea water (pH = 8.2 to 8.3, in the situations concerned). This fact, taken together with observations I have recently made upon *Chromodoris zebra*, seems sufficient to show that the reaction of protoplasmic fluids need not be regarded as a

TABLE II.

Indications as to Intracellular Acidity in Animal Tissues.

Animal.	Tissue.	Color.	Indicated acidity, pH.
Sponge A.	Body substance.	Yellow.	<6.0
	Thin projections.	Purple.	8.0±?
Sponge B.	Cortex.	Brown.	8.0±
	Choanosome.	Scarlet.	<7.0
Mellita.	Integument.	Brown.*	<7.5
Chromodoris.	Integument.	Blue.	>5.6, <7.6

* Due to a separate pigment; "colorless" so far as the alkali-greening material is concerned: milk-white in young individuals.

constant, invariable property. In several cases I have discovered young or medium sized active individuals of this nudibranch in which some ordinarily blue portions of the integument were bright pink. Similarly, the slight variations in the blue to purple hue of the mantle tissues of *Chromodoris* further suggest that the normal reaction of the cells concerned is acid rather than alkaline. The few (large) specimens I have been able to discover apparently undergoing natural death in the field, were red in the parts colored by the indicator, pointing to decidedly acid condition in connection with natural death.[3] Regenerating portions of

[3] Experiments with *Chromodoris* yield no support to the idea that death at high temperatures is due to acid intoxication.

the mantle of *Chromodoris* are deep blue, rather than purple, in all individuals. Whether this signifies merely a more active exchange of materials at the surface of such regenerating areas, I cannot say; there is no indication of this character in the regeneration of *Mellita* (cf. Crozier, 1918*b*). I have sought, but without success, for any suggestion in these pigmented animals of higher alkalinity in the protoplasmic juices of young, rapidly growing individuals.

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Crozier, 1918*b*, On regeneration and the re-formation of lunules in *Mellita*, *Am. Nat.*, lii, in press.

Harvey, E. N., 1914, Cell permeability for acids, *Science*, xxxix, 947–949.

OBSERVATIONS ON THE SIGNIFICANCE OF GLYCOLLIC ACID, GLYOXAL, GLYCOL ALDEHYDE, AND AMINO-ALDEHYDE IN INTERMEDIARY METABOLISM.

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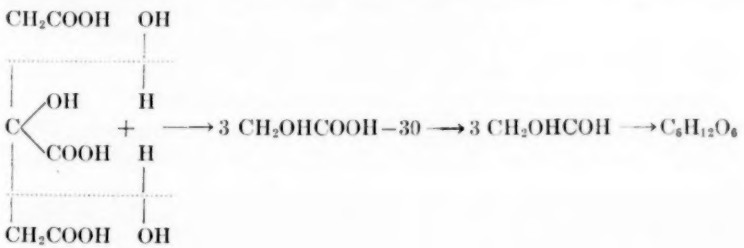
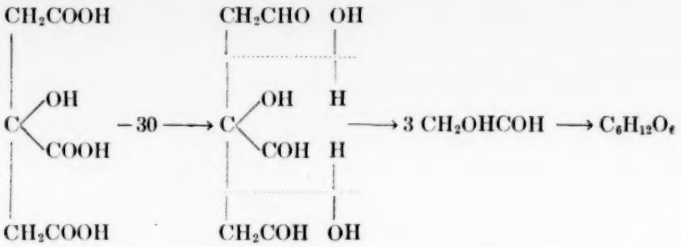
The experiments to be reported in this paper were performed 4 years ago. It had been the author's intention to repeat some of them and to add others that would serve to test the truth of the hypotheses to be presented. A number of circumstances operated to prevent this. The possibility of the resumption of these investigations being now rather remote, it was thought advisable to put on record such results as have been obtained.

The investigation developed as an attempt to discover the stages in the apparent conversion of citric acid into glucose in a phlorhizinized patient and in one with diabetes mellitus.[1] The amount of "extra glucose" excreted after the administration of citric acid indicates the conversion of all six carbon atoms of the citric acid into glucose. Examination of the reactions that might yield such a result leads to the following alternatives.



or

[1] Greenwald, I., *J. Biol. Chem.*, 1914, xviii, 115.



The first series of reactions is improbable for the administration of glycollic acid does not increase the excretion of glucose in the phlorhizinized dog.[2, 3] These observations have been confirmed in this laboratory (Table I). It is, of course, possible that normally glycollic acid is reduced as rapidly as it is formed, while when administered it is present in too high concentration and is either oxidized or excreted unchanged. Pohl[4] found neither glycollic nor oxalic acid in the urine of a dog after the administration of glycollic acid. But Dakin[5] did observe an increased excretion of oxalic acid. Unfortunately the fate of the glycollic acid administered in my experiments was not determined. But whatever it may have been, it could not support the view that glycollic acid is an intermediary in the formation of glucose.

In the other series of reactions, citric trialdehyde appears as an intermediate product. This substance has not yet been described, and attempts to prepare it were unsuccessful.

Both series of reactions call for the formation of glycol aldehyde. This substance is the simplest of all the sugars and has naturally been thought to be of physiological significance. The evidence is not conclusive. Paul Mayer[6] observed that the administration of glycol aldehyde to rabbits resulted in a marked glycosuria, but since this phenomenon in rabbits is readily induced by a variety of means, this finding cannot be accepted as good evidence of the formation of glucose from gylcol aldehyde. Smedley[7] reported

[2] Baer, J., and Blum, L., *Beitr. Chem. Physiol. u. Path.*, 1907, x, 80.
[3] Ringer, A. I., Personal communication to the author.
[4] Pohl, J., *Arch. exp. Path. u. Pharmakol.*, 1896, xxxvii, 413.
[5] Dakin, H. D., *J. Biol. Chem.*, 1907, iii, 57.
[6] Mayer, P., *Z. physiol. Chem.*, 1903, xxxviii, 135.
[7] Smedley, I., *J. Physiol.*, 1912, xliv, 203.

that liver tissue added to a solution of glycol aldehyde caused a marked diminution in reducing power. Since both substances have approximately the same reducing action upon alkaline copper solutions, this observation cannot be regarded as evidence of the formation of glucose from gylcol aldehyde. It is much more likely to be due to the action of the enzyme "aldehydemutase" described by Parnas[8] which induces, or accelerates, a Cannizzarro reaction by which one molecule of an aldehyde is oxidized to the acid at the expense of another, which is reduced to the alcohol. According to Parnas and Baer,[9] glycol aldehyde gave rise to glycogen in the perfused turtle liver, and Barrenscheen[10] found that if glycol aldehyde were added to the blood used for perfusing the liver of a phlorhizinized dog there was an increase in the amount of a dextrorotatory substance in the blood. It is interesting to note that Parnas and Baer did not observe an increase in the glycogen content of the liver after perfusion with blood containing glycollic or glyoxylic acids.

Woodyatt[11] gave glycol aldehyde to three phlorhizinized dogs but failed to observe an increase in the excretion of glucose or in the ratio of glucose to nitrogen in the urine. Later experiments reported by Sansum and Woodyatt[12] seemed to indicate a formation of glucose but Sansum and Woodyatt avoided a definite conclusion.

Cremer[13] gave glycol aldehyde (obtained by the decomposition of dihydroxymaleic acid in water) to phlorhizinized dogs and observed a rise in the ratio of glucose to nitrogen, but this continued for several periods with an accompanying retention of nitrogen. The experiment was, therefore, inconclusive.

In my earlier experiments, a similar crude gylcol aldehyde solution was employed. The first two experiments (Table II) were successfully carried out. There was a well marked rise in the excretion of glucose and in the ratio of glucose to nitrogen in the urine. In the three following experiments, however, results simi-

[8] Parnas, J., *Biochem. Z.*, 1910, xxviii, 274.
[9] Parnas, J., and Baer, J., *Biochem. Z.*, 1912, xli, 386.
[10] Barrenscheen, H. K., *Biochem. Z.*, 1914, lviii, 277.
[11] Woodyatt, R. T., *J. Biol. Chem.*, 1914, xvii, p. xxix.
[12] Sansum, W. D., and Woodyatt, R. T., *J. Biol. Chem.*, 1914, xvii, 521.
[13] Cremer, M., *Beitr. Physiol.*, 1915, i, 255.

lar to Cremer's were obtained.[14] It was then determined to use a purer glycol aldehyde, obtained by treating the solution obtained as above with calcium carbonate, filtering, evaporating *in vacuo*, precipitating the calcium salt of an acid (nature unknown) with absolute alcohol, and finally evaporating off the alcohol under diminished pressure. The resulting syrup was dissolved in water and the aldehyde content determined by reduction of Fehling's solution. This material yielded one positive experiment (Table III) and three that were inconclusive because of the retention of nitrogen in the after periods.

In all subsequent experiments, crystalline glycol aldehyde, prepared by the decomposition of dihydroxymaleic acid in pyridine,[15] was employed. With this product, and following the usual technique, three positive experiments were obtained (Table IV).

The work had reached this stage when Sansum and Woodyatt[16] published their observations on the action of narcotic drugs in phlorhizin diabetes. Although glycol aldehyde had manifested no narcotic properties, it was believed advisable to perform some experiments upon animals previously prepared with adrenalin, as described by Sansum and Woodyatt. Such animals proved to be much more profoundly affected by the toxic action of glycol aldehyde than those not so treated, and, after a few experiments with 12 hour periods, in which no satisfactory after periods could be obtained, it was decided to employ 6 hour periods. Of four experiments in which the nitrogen retention that presaged death did not occur until after at least one after period, three were positive and one was negative. It was thought possible that there had been a retention of the glucose as glycogen. Accordingly, 0.3 mg. of adrenalin was injected, but without effect. This was the only experiment out of a total of twenty-one (nine with adrenalin) in which the administration of glycol aldehyde was not followed by a rise in the glucose: nitrogen ratio. In the experiments which are not reported in detail, this high ratio continued and was accompanied by a marked diminution in the excretion of nitrogen. The dogs died within 2 or 3 days. At autopsy, a severe acute nephritis was demonstrated.

[14] This work was completed before Cremer's paper appeared.

[15] Fenton, H. J. H., *J. Chem. Soc.*, 1905, lxxxvii, 817.

[16] Sansum and Woodyatt, *J. Biol. Chem.*, 1915, xxi, 1.

The experiments may not be conclusive, but they do make it highly probable that glycol aldehyde may be converted into glucose in the animal body.

We may now turn to the question of the formation of glucose from glycine. The results of Ringer and Lusk[17] and of Csonka[18] seem to make it evident that both carbon atoms of glycine appear as glucose. This is denied by Cremer and his pupils.[13, 19] Their conclusions are based in part upon experiments with acetyl glycine and glycine ester hydrochloride. In their calculations, they assume that these substances are completely decomposed into glycine and acetic acid, or alcohol. This may be true, but evidence is lacking. Cremer also reports some experiments in which glycine was fed to phlorhizinized dogs. The results of these experiments appear to agree with his idea of the conversion of three atoms of carbon of every two molecules of glycine into glucose. But it seems rather questionable that all the glycine fed was metabolized. The amounts given were large, so large that some of the animals vomited. The glycine was given as the solid, mixed with lard. Almost all the conditions for defective absorption were present: large dosage, high concentration, and mixture with a substance that is itself rather poorly absorbed. The possible action of the intestinal flora is also to be considered. Even if absorbed, a small part may have been excreted unchanged, or in some form other than glucose, in the urine. Only a very small amount unchanged in the urine would affect the glucose : nitrogen ratio very markedly.

Dakin and Dudley[20] have shown that amino-acids *in vitro* readily lose ammonia with the formation of an hydroxy ketone and have advanced the hypothesis that this process plays a part in the metabolism of amino-acids. In the case of glycine, this reaction would yield glyoxal. But in the presence of the glyoxalase of the tissues, this should be immediately converted into glycollic acid. In fact, Dakin and Dudley[21] have found that glyoxal perfused through a surviving liver is converted into

[17] Ringer, A. I., and Lusk, G., *Z. physiol. Chem.*, 1910, lxvi, 106.
[18] Csonka, F. A., *J. Biol. Chem.*, 1915, xx, 539.
[19] Folger, F., *Beitr. Physiol.*, 1915, i, 187.
[20] Dakin, H. D., and Dudley, H. W., *J. Biol. Chem.*, 1913, xv, 127.
[21] Dakin and Dudley, *J. Biol. Chem.*, 1914, xviii, 29.

glycollic acid. This, as we have seen, is probably not a precursor of glucose.

The question of the formation of glucose from glyoxal was also tested by direct experiment with negative results. Freshly distilled glyoxal was employed. None of the animals showed an increase in the glucose : nitrogen ratio during the experimental period (Table VI). The following periods developed a sequence of toxic symptoms (high ratios, nitrogen retention, etc.) similar to those observed after the administration of glycol aldehyde, *but the excretion of glucose and the ratio of glucose to nitrogen during the experimental period was not affected.*

The fact that glycine may be excreted in such large amounts in the form of hippuric acid after the administration of benzoic acid would seem to call for a relatively simple reaction for its formation. In the light of our present knowledge of the fate of amino-acids, it is almost inconceivable that such amounts of glycine should be directly derived from other amino-acids. It seems much more probable that the reaction glycine → glucose also occurs in the reverse direction. A possible sequence is glycine ⇆ amino-aldehyde ⇆ glycol aldehyde ⇆ glucose. This reaction for the formation of glucose from glycine appears to have been first suggested by Magnus-Levy.[22]

Kikkoji and Neuberg[23] fed amino-aldehyde to a rabbit for several days and collected the urine. They found no optically active or reducing substance in the urine. They isolated a small quantity of pyrazine and concluded that amino-aldehyde may be oxidized to pyrazine in the body. But the largest part of the amino-aldehyde was either completely oxidized or converted into some other derivative.

I have performed one experiment with amino-aldehyde with positive results. It did give rise to "extra glucose." The urine was tested for unchanged amino-aldehyde and for pyrazine with negative results.[24] Positive experiments do not add greatly to the strength of the hypothesis for it may always be said that the

[22] Magnus-Levy, A., in Oppenheimer, C., Handb. Biochemie, Jena, 1911, pt. i, 354.

[23] Kikkoji, T., and Neuberg, C., *Biochem. Z.*, 1909, xx, 463.

[24] Wolff, L., *Ber. chem. Ges.*, 1893, xxvi, 1830. Gabriel, S., and Pinkus, G., *ibid.*, 1893, xxvi, 2207.

aldehyde was oxidized to glycine and that this gave rise to glucose in the usual manner. Negative experiments would disprove the hypothesis. Lack of material prevented repetition of the experiment at that time. It is my purpose to repeat it when opportunity offers.

TABLE I.

Experiments with Glycollic Acid, 12 Hr. Periods.

Period.	Glucose.		Nitrogen.	G:N	Remarks.
	Benedict.	Polariscope.			
	gm.	*gm.*	*gm.*		
I	16.40	16.18	4.587	3.57	
II	13.87	13.23	4.487	3.09	
III	**14.45**	**14.23**	**4.187**	**3.42**	Wt., 6.36 kg. 4.15 gm. glycollic acid (as sodium salt).
IV	11.21	11.54	3.543	3.16	
V	10.21	9.81	3.154	3.24	
VI	**10.51**	**10.73**	**3.297**	**3.19**	5.26 gm. glycollic acid (as sodium salt).
VII	9.59	9.96	2.943	3.26	
VIII	9.30	9.54	2.977	3.12	
I	19.32	19.82	6.363	3.04	
II	18.51	18.35	6.120	3.03	
III	**21.45**	**21.96**	**7.009**	**3.22**	Wt., 10.9 kg. 6.53 gm. glycollic acid (as sodium salt).
IV	18.84	18.30	5.680	3.19	
V	**17.83**	**17.48**	**6.067**	**2.94**	6.57 gm. glycollic acid (as sodium salt).
VI	18.38	17.36	5.556	3.31	
VII	15.22	14.45	4.958	3.07	
VIII	14.53	14.62	4.551	3.19	

EXPERIMENTAL.

The technique of the experiments was that usually employed in this laboratory. The animals received no food and were daily injected, subcutaneously, with 1 gm. of phlorhizin suspended in cottonseed oil. The urine was collected by catheter in 6 or 12 hour periods, the bladder being washed with 2 per cent sterile boric acid solution. The substance to be tested was injected subcutaneously, in aqueous solution, at intervals of from 30 to 60 minutes during the first half of the experimental period.

Glucose was determined by Benedict's method[25] or by the Allihn method, weighing as cupric oxide. The values so obtained were checked by polarization before and after fermentation and by fermentation. Acetone and β-hydroxybutyric acid were determined by Shaffer's method.[26]

The glycollic acid was prepared by heating a solution of chloroacetic acid with calcium carbonate, recrystallizing the calcium

TABLE II

Experiments with Crude Glycol Aldehyde. 12 Hr. Periods.

Period.	Glucose.		Nitrogen.	G:N	Acetone.	β-Hydroxybutyric acid.	Remarks
	Benedict.	Polariscope.					
	gm.	*gm.*	*gm.*		*gm.*	*gm.*	
I	13.9	13.2	3.50	3.96	0.0064	0.065	
II	11.8	12.3	3.39	3.47	0.015	0.071	
III	**14.4**	**13.5**	**3.35**	**4.30**	**0.026**	**0.060**	Wt., 5.05 kg. 2.43 gm. glycol aldehyde; "extra glucose" 1.66 gm.
IV	11.1	10.5	2.80	3.98	0.016	0.049	
I	14.6	15.0	3.99	3.65	0.133	1.10	
II	12.2	11.4	3.74	3.25	0.184	1.15	
III	**14.7**	**13.0**	**3.58**	**4.12**	**0.192**	**0.79**	Wt., 6.95 kg. 3.11 gm. glycol aldehyde; "extra glucose" 2.53 gm.
IV	11.23	11.5	3.38	3.33	0.104	0.76	

salt several times, and finally decomposing it with the calculated quantity of oxalic acid.

Glycol aldehyde was prepared by the decomposition of dihydroxymaleic acid, which was prepared by the oxidation of tartaric acid with hydrogen peroxide.[27] The crude dihydroxymaleic acid was dried *in vacuo*, and purified by solution in absolute methyl alcohol and precipitation with water. This acid was then heated, either in water or pyridine, as already indicated.

[25] Benedict, S. R., *J. Biol. Chem.*, 1911, ix, 57.
[26] Shaffer, P. A., *J. Biol. Chem.*, 1908–09, v, 211.
[27] Nef, J. U., *Ann. Chem.*, 1907, ccclvii, 290.

TABLE III.

Glycol Aldehyde Purified with $CaCO_3$, Alcohol, Etc.

Period.	Glucose.		Nitrogen.	G : N	Remarks.
	Benedict.	Polariscope.			
	gm.	*gm.*	*gm.*		
I	11.9	11.8	3.56	3.35	
II	12.1	12.2	3.79	3.21	
III	**16.9**	**16.2**	**4.18**	**4.05**	Wt., 6.86 kg. 2 gm. glycol aldehyde; "extra glucose" 2.9 gm.
IV	11.2	10.7	3.21	3.50	

TABLE IV.

Experiments with Crystallized Glycol Aldehyde. 12 Hr. Periods.

Glucose.			Nitrogen	G : N	Acetone.	β-Hydroxybutyric acid.	Remarks.
Benedict.	Polariscope.	Fermentation.					
gm.	*gm.*	*gm.*	*gm.*		*gm.*	*gm.*	
20.7	20.8	20.9	5.79	3.57			
16.8	16.8	16.5	4.67	3.60			
16.9	16.6	16.5	4.98	3.40	0.164	0.739	
24.7	**23.4**	**24.7**	**6.07**	**4.06**	**0.210**	**1.118**	Wt., 8.0 kg. 4 gm. glycol aldehyde; "extra glucose" 3.47 gm.
18.4	17.6	18.1	5.01	3.67	0.247	1.077	
16.1	16.3	16.7	4.28	3.75			
17.8	17.8	17.0	4.65	3.83			
15.7	15.7	15.3	4.81	3.27	0.131	0.770	
23.3	**23.1**	**22.8**	**5.68**	**4.11**	**0.145**	**0.722**	5.05 gm. glycol aldehyde; "extra glucose" 4.54 gm.
18.7	18.3	18.9	5.45	5.35	0.179	0.954	
19.9	19.7	19.4	5.65	3.52			
21.1	21.3	21.0	5.93	3.55	0.040	0.192	
28.8	**28.2**	**28.8**	**6.74**	**4.28**	**0.035**	**0.202**	Wt., 7.74 kg. 4.29 gm. glycol aldehyde; "extra glucose" 4.25 gm.
22.0	21.6		5.85	3.75	0.052	0.243	

TABLE V.

Experiments with Crystallized Glycol Aldehyde upon Dogs Receiving Adrenalin. 6 Hr. Periods.

Glucose.			Nitrogen.	G:N	Adrenalin.	Remarks.
	Polariscope.	Fermentation.				
gm.	*gm.*	*gm.*	*gm.*		*mg.*	
Allihn.						
14.5	14.3	14.0	4.08	3.55	0.25	12 hrs.
8.40	8.75	8.7	2.57	3.27	0.30	
9.81	**9.67**	**9.6**	**2.22**	**4.42**		Wt., 5.7 kg. 2.07 gm. glycol aldehyde; 2.51 gm. "extra glucose."
8.12	8.13	8.1	2.46	3.31		
5.71	5.55	5.75	1.51	3.80		
Benedict.						
14.0	13.9	14.0	4.60	3.04	0.50	
15.1	15.7	15.6	5.08	2.97	0.50	
30.3	31.1	30.2	9.61	3.15		12 hrs.
16.1	**16.7**	**16.0**	**5.13**	**3.20**		Wt., 13.7 kg. 4.25 gm. glycol aldehyde; no "extra glucose."
14.6	14.6		4.45	3.28		
13.5	13.6		3.92	3.45	0.50	
Allihn.						
13.9	13.6		3.94	3.61	0.35	12 hrs.
7.41	7.16		2.32	3.20	0.25	
6.67	6.40	6.6	2.19	3.05		
11.85	**11.90**	**11.6**	**2.50**	**4.75**		Wt., 8.90 kg. 2.42 gm. glycol aldehyde; 3.0 gm. "extra glucose."
9.11	8.75	9.0	2.61	3.49		
6.73	6.96	6.75	2.04	3.30		
8.66	8.20	8.75	2.48	3.49		
Allihn.						
9.98	10.3	10.2	3.06	3.26	0.30	
9.88	10.2	9.9	3.01	3.29	0.40	
12.38	**12.6**	**12.2**	**2.88**	**4.30**		Wt., 10.77 kg. 3.06 gm. glycol aldehyde; 2.74 gm. "extra glucose."
10.50	10.7	10.5	3.01	3.50		
8.69	8.35	8.7	2.60	3.34		

TABLE VI.

Experiments with Glyoxal upon Dogs Receiving Adrenalin. 6 Hr. Periods.

Glucose.			Nitrogen.	G:N	Adrenalin.	Remarks.
Allihn.	Polariscope.	Fermentation.				
gm.	*gm.*	*gm.*	*gm.*		*mg.*	
12.1		12.1	4.23	2.85	0.50	12 hrs.
7.35		7.6	2.77	2.65	0.50	
6.89		6.8	2.59	2.66		
4.46	**4.58**	**4.8**	**1.58**	**2.83**		Wt., 7.2 kg. 3.85 gm. glyoxal.
8.30	8.27	8.0	2.63	3.15	0.50	7.3 hrs.
7.14	7.14	7.4	2.23	3.21	0.50	
5.42	**5.03**	**5.5**	**1.78**	**3.04**		2.30 gm. glyoxal.
5.98	5.88	5.8	1.50	3.99		
4.79			1.03	4.63		
11.24		11.2	2.87	3.91	0.50	
8.96		9.0	2.70	3.32		
4.56		4.6	1.27	3.59		4.26 gm. glyoxal.

TABLE VII.

Amino-Aldehyde. 12 Hr. Periods.

Glucose. Benedict.	Nitrogen.	G:N	Remarks.
gm.	*gm.*		
31.7	9.26	3.42	24 hrs.
18.6	5.74	3.24	
16.3	4.81	3.39	
12.4	**3.28**	**3.79**	3 gm. amino-aldehyde; 4 gm. "extra glucose."
6.9	2.30	3.00	

In the latter case, the pyridine was distilled off and the glycol aldehyde distilled at about 4 mm. pressure and then freed from traces of pyridine by standing over concentrated sulfuric acid.

Glyoxal was distilled just before using by distilling a mixture of polymeric glyoxal and phosphorus pentoxide into ice water.[28]

[28] Harries, C., and Temme, P., *Ber. chem. Ges.*, 1907, xl, 165.

The distillates were very slightly acid. The glyoxal content was estimated either by precipitation with *p*-nitrophenylhydrazine and weighing or by titration with standard acid and alkali. When heated with a slight excess of alkali, glyoxal is quantitatively converted into glycollic acid. The excess alkali may then be titrated. The two methods gave closely agreeing results; thus, 2.19 per cent by titration and 2.12 per cent by precipitation, and in another case, 3.80 per cent and 3.66 per cent, respectively.

The amino-aldehyde was prepared by hydrolysis of the acetal (Kahlbaum's) with hydrochloric acid and evaporation *in vacuo*.[29] The amount was calculated from that of the acetal used.

[29] Fischer, E., *Ber. chem. Ges.*, 1893, xxvi, 92.

A RAPID MICRO METHOD FOR THE DETERMINATION OF PHOSPHATE AND TOTAL PHOSPHORUS IN URINE AND STOOLS.

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The method is a colorimetric one and depends upon the precipitation of phosphate by uranium. The precipitated phosphate is dissolved in acid and the color developed by the addition of potassium ferrocyanide is compared with that produced by the same reagent added to a standard uranium phosphate solution.

Solutions Required.

1. Standard Phosphate Solution.—Dissolve 1.918 gm. of recrystallized potassium phosphate (KH_2PO_4) in distilled water and make up to 100 cc. 1 cc. of this solution equals 0.01 gm. of P_2O_5. Any other pure salt of phosphoric acid may be used for preparing the standard solution.

2. Uranium Acetate Solution.—(*A*) 10 gm. of uranium acetate dissolved in water and made up to 300 cc. (*B*) 10 cc. of solution A made up to 100 cc. with distilled water.

3. Acetate Solution.—Sodium acetate 100 gm. and glacial acetic acid 30 cc., made up to 1 liter.

4. Potassium Ferrocyanide Solution.—10 per cent in distilled water.

5. Cochineal Tincture.

6. Standard Uranium Phosphate Solution.—Add 20 cc. of water and 10 cc. of acetate solution to 20 cc. of the standard phosphate solution and heat to boiling. Add an excess of uranium acetate solution A (about 45 cc.) while the mixture is boiling. Add 30 cc. of 95 per cent alcohol and allow precipitate to settle. Filter and wash the precipitate with 10 per cent alcohol until the last

washing fails to change color when a drop of cochineal tincture is added. It is unnecessary to remove the precipitate adherent to the sides and bottom of the beaker. Remove filter paper from the funnel and return to the beaker; add exactly 100 cc. of 10 per cent hydrochloric acid (specific gravity 1.050), cover with watch-glass, and agitate until precipitate is completely dissolved. Do not heat beaker. When completely dissolved transfer liquid to a 200 cc. volumetric flask and make up to mark with distilled water. For color comparison the solution is diluted one to twenty and 10 cc. are used. This amounts to 0.0005 gm. of P_2O_5.

Determination of Inorganic Phosphate in Urine.[1]

Determination of Amount of Urine to Be Used for Analysis.— Put a drop of urine and a drop of acetate solution in a test-tube and a drop[2] of uranium acetate solution B and about 3 cc. of water. Boil for 10 seconds. Add three drops of cochineal tincture, noting color changes. The color sought is purple and neither red nor green.

With an excess of urine in proportion to the uranium solution the color is red; with an excess of uranium the color is green. If the color is green, more urine is used and *vice versa*. A few trials show the relative proportions in which the urine and the uranium solutions must be used.

The following table indicates roughly the proportion for various urines:

	Urine (drops).	Uranium solution (drops).
Adult	1	20–30
Bottle-fed infant	1	2
Breast-fed infant*	5	1

* One may readily test by adding the uranium solution to a drop of infant's urine whether the child is receiving cow's milk or human milk. If one drop of the uranium solution produces a green color, the urine is from a breast-fed child, provided always that the child is receiving milk of some kind.

[1] If the urine is cloudy from phosphates enough diluted acetic acid should be added to bring the phosphate into solution.

[2] The droppers used for the urine and the uranium solution should deliver drops of the same size.

If we express the number of drops of urine taken by a and the number of drops of uranium solution by b, then the number of cc. of urine to be used should be $\frac{a}{b}$ cc. Thus with the adult we would use one-twentieth to one-thirtieth of 1 cc., with the bottle-fed infant about 0.5 cc., and with the breast-fed infant 5 cc. In order to use such small amounts of urine, it is of course advisable to dilute an accurately measured quantity of urine and to take a portion of this.

Method of Analysis.—Measure the proper amount of urine as determined above into a small beaker. Add 5 to 7 drops of acetate solution and not more than 1.5 cc. of uranium solution B. Boil and add 95 per cent alcohol, in amount about equivalent to one-third the total amount of liquid. Filter through a small paper and wash precipitate with 10 per cent alcohol until the last washing shows no change of color with cochineal tincture. Three or four washings are usually sufficient. Return paper to beaker and add exactly 5 cc. of 0.5 per cent HCl to dissolve precipitate. Cover with watch-glass, warm gently for not more than a minute, and then cool. Rinse solution into a 50 cc. volumetric flask bringing up to about 30 cc. In another 50 cc. flask put exactly 10 cc. of the diluted uranium phosphate standard described above and add approximately 20 cc. of water. Add 5 cc. of the ferrocyanide solution to each flask. A reddish brown color develops immediately. Fill each flask to mark and mix. Compare the colors at once in a Duboscq colorimeter, setting the standard at 20 mm. The color of the unknown solution should not vary by more than 25 per cent from that of the standard. The calculation is as follows:

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}}\ 0.0005 \text{ gm.} = P_2O_5 \text{ in amount of urine taken.}$$

or

$$\frac{\text{Reading of standard}}{\text{Reading of unknown} \times 2 \text{ mg.}} = P_2O_5 \text{ in amount of urine taken.}$$

A number of factors may affect the intensity of the color.[3] One of these is the concentration of HCl but this is the same in

[3] Gibson, R. B., and Estes, C., *J. Biol. Chem.*, 1909, vi, 356.

both control and test solutions. Heat is another factor so that each sample must be at the same temperature before the ferrocyanide solution is added.

Total Phosphorus in Urine and Stools.

Urine.—Use the same amount of urine as for inorganic phosphates. Put in a large test-tube with 0.5 cc. of concentrated sulfuric acid and add 0.5 cc. of concentrated nitric acid, drop by drop, heating in the meantime. A colorless solution should be obtained and the yellow fumes cease to come off when digestion is complete. A short time is required for this. Cool and dilute cautiously with water and neutralize with 10 per cent NaOH using methyl orange as an indicator. When just neutral, warm to remove any great excess of ammonia that may be present. (A slight amount of ammonia does no harm.) Add 1 per cent acetic acid, drop by drop, until the liquid is faintly acid as indicated by a pink color. Transfer to a beaker and proceed exactly as described above for the determination of inorganic phosphates.

Stools.—At the outset it is impossible to determine the optimum amount of stool to be taken. An excess should be taken; for instance, 1 per cent or more of the total 24 hour stool. Digest in a large test-tube or a small Kjeldahl flask with sulfuric and nitric acids as described above for urine. Proceed as for urine to the point where acetic acid has been added until a faint pink color is obtained. Then transfer the solution to a 50 cc. volumetric flask and fill to the mark with water. Estimate as in the case of inorganic phosphate in urine the amount of the solution to be used and proceed as for the determination of inorganic phosphates in urine.

Inorganic Phosphates in Stools.

Use an excess of stool. Mix with 2 cc. of water and add an equal volume of concentrated hydrochloric acid. Mix thoroughly and allow to stand 10 minutes to dissolve the inorganic phosphate present. Filter and wash precipitate several times with small amounts of 5 per cent HCl (specific gravity 1.025). Neutralize with 10 per cent NaOH using methyl orange as an indicator. Add 1 per cent acetic acid till faintly acid (pink color) and proceed as for total phosphorus in stools.

In the analysis of pure phosphate solution I have been able to determine the amount of inorganic phosphate with an average error not exceeding 2 per cent. In the analysis of urine and feces the method described above has yielded results differing by not more than 2 per cent from those obtained by the ordinary gravimetric methods.

THE SCURVY OF GUINEA PIGS.

I. THE EXPERIMENTAL DIETARY.

By ALFRED F. HESS and LESTER J. UNGER.

(*From the Bureau of Laboratories, Department of Health, New York.*)

(Received for publication, June 19, 1918.)

In this paper we wish to discuss the results of an investigation on scurvy as it is manifested in the guinea pig. It should always be understood that studies made upon animals cannot be accepted unreservedly in the domain of human pathology, but that they are merely tentative and introductory in nature, and must await confirmation by clinical medicine. This deserves particular emphasis in connection with the disorder which we have under consideration.

In experiments on the scurvy of guinea pigs various diets have been fed. Most of the workers have followed the classical investigation of Holst and Frölich (1) and employed a diet of oats and water. More recently, however, an unlimited quantity of milk has been added to this dietary, especially in the studies reported by Jackson and Moore (2), and by McCollum and Pitz (3). The disadvantage of the latter dietary for the study of scurvy would seem apparent when we consider that milk possesses some antiscorbutic power, so that if we allow it *ad libitum* we cannot know how much antiscorbutic foodstuff is consumed. The essential importance of quantity in this connection is brought out by some recent experiments (4) which show that 50 gm. of milk daily is insufficient to protect guinea pigs from scurvy, whereas 100 gm. is quite sufficient. In the following experiments, which have been reported elsewhere in abstract (5), we have mainly employed a diet consisting of hay, oats, and water, and by means of various tests have attempted to analyze this dietary and to note the effect of the addition of other foodstuffs.

It would seem that the confused state in which this subject stands at present is due largely to the fact that various investi-

gators have not made use of the same diet, and that they have not controlled their studies by means of pathological examinations. All work on this subject should be checked by careful histological examination of the bones. As far as we are aware there are changes in the epiphyseal ends of the bones which are typical of scurvy and differ from those which occur in other diseases of the bony system. It is, therefore, imperative that such lesions be found before we take it for granted that we are really dealing with scurvy. We emphasize this fact because we have encountered macroscopic lesions at autopsy which we considered characteristic of true guinea pig scurvy (hemorrhages into the costochondral junctions of the ribs and swelling of the joints) but which later microscopic examination proved to resemble rickets. The bones of the animals referred to in this study showed the typical "frame work" marrow at the epiphyseal junction, a marrow poor in cells and rich in connective tissue; it did not show the osteoid tissue always associated with rickets.

We have illustrated our results by means of a series of charts, although we believe it to be impossible to portray scurvy graphically in this way, in view of the fact that there is no true parallelism between the progress of scurvy and deviations in weight. It is a fact that most animals lose in weight with the development of scurvy. The charts which are appended as well as those of others clearly show, however, that the guinea pigs may gain somewhat and nevertheless develop scurvy, and on the other hand may lose or remain stationary in weight and be free from all scorbutic signs. We have attempted to make up for this defect by adding, in many cases, a curve representing the clinical course of the scurvy. Although this is computed in an empirical way, it gives a truer picture of the course of the disease than the weight chart. A maximum count of 35 was constituted, representing the greatest degree of clinical scurvy. This included three grades of beading of the ribs, four grades of tenderness of each knee joint and wrist, and four grades of swelling of each of these joints.

It is apparent from the clinical curve of Chart 1 that an animal may have developed a marked degree of scurvy (20 points) but nevertheless have gained rather than lost in weight during the period of observation. This chart likewise shows that the signs of scurvy continue to increase for some days after the antiscor-

butic is added to the dietary; in this instance for 7 days, showing that there is a latent therapeutic period. A similar phenomenon is seen to occur when the orange juice was discontinued, the scurvy continuing to improve for a period of about a week.

The following deductions may be made from our experiments which cover the use of several hundred guinea pigs, and are partially illustrated by the charts which accompany this paper.

It will be seen from Chart 1, that guinea pigs develop scurvy on a diet of hay, oats, and water given *ad libitum*. The hay was

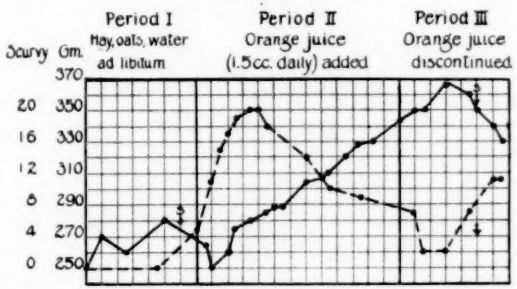

CHART 1. Hay, oats, and water *ad libitum* during Period I resulted in a lack of gain in weight, and in a development of scurvy. During Period II, 1.5 cc. of orange juice were given daily, and as will be seen there was a marked gain in weight and disappearance of the scorbutic signs, although, as is generally the case, they contrive to develop for a short while after an antiscorbutic is given. When the orange juice was discontinued in the third period, the weight once more fell and the scurvy redeveloped.

The broken line represents the course of the scurvy, and is a composite formed on the basis of the aggregate of the scorbutic signs. Each square represents a 2 day interval.

given to supply the ash of the green fodder. It will be seen, however, from a later experiment that it has additional value, probably owing to its roughage, and that it is not replaceable in the dietary by its ash (Chart 8). This diet (as has been reported elsewhere) has the advantage of bringing about scurvy in all animals without exception, and in leading to scorbutic signs in guinea pigs weighing 200 to 300 gm. within a period of 10 to 20 days. It thus permits of a quantitative estimate of the influence of diet or other factors in relation to scurvy. It will be noted (Chart 1) that the mere addition of 1.5 cc. of orange juice daily

are sufficient to bring about a marked and steady gain in weight. This is brought out again by Chart 2. It is not, however, suggested as a complete diet for permanent normal growth.

When animals were put on a straw instead of a hay bedding, they did not do so well (Chart 3). It is difficult to state how

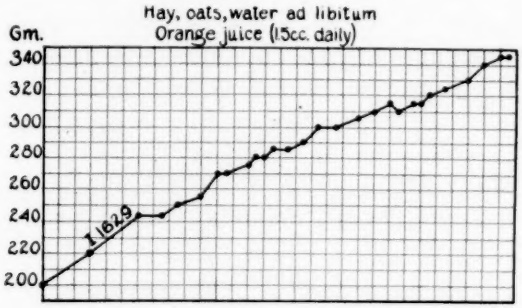

CHART 2. Showing the steady gain in weight on a diet of hay, oats, and water *ad libitum*, with the daily addition of but 1.5 cc. of orange juice to the diet.

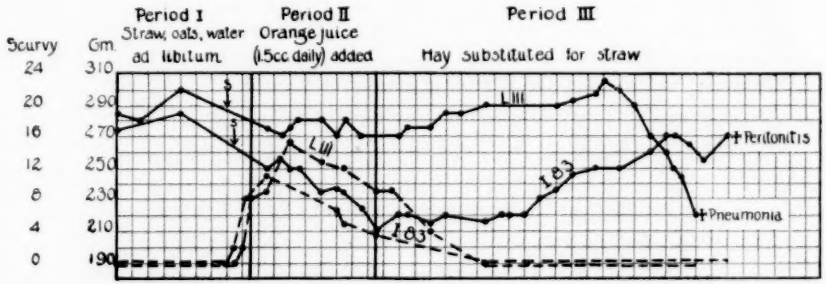

CHART 3. Showing the value of hay in the dietary. It also illustrates a fact which was frequently encountered in these experiments; that after an animal has once had scurvy, although all signs may disappear, a considerable period of subnormal gain in weight may follow.

much straw they consumed. They were observed, however, to nibble it from time to time. Orange juice added to this dietary was not able to cause a normal gain in weight, although it cured the animals of scurvy. As this dietary of hay, oats, and water, although it contains a sufficient supply of inorganic salts, and of the

water-soluble vitamine, as well as roughage, is deficient in fat protein and fat-soluble vitamine, it was decided to add a foodstuff which would compensate for this deficiency. Accordingly egg (both yolk and white) was given in the amount of 9 gm. daily to each guinea pig; the egg was hard boiled and chopped fine, and the

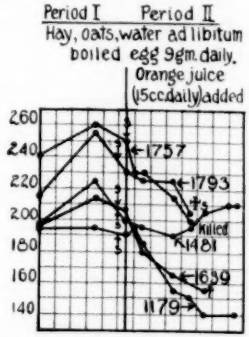

CHART 4. Hay, oats, water *ad libitum*, plus 9 gm. of hard boiled egg daily (yolk and white). Of these five guinea pigs four developed scurvy after 10 days, although they had all gained slightly in weight during this period. The other showed signs after 11 days. On the addition of orange juice the signs of scurvy disappeared in all but one animal.

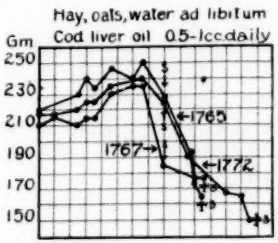

CHART 5. The addition of cod liver oil to the regular diet of hay, oats, and water *ad libitum*, did not delay the onset of scurvy, although it seemed to cause a temporary gain in weight.

animals took it well. It was found, however, that this addition did not delay the onset of the disease. Of the five guinea pigs shown in Chart 4, three developed scurvy after 10 days, and the other one 1 day later. Practically the same result was noted when cod liver oil in doses of 0.5 to 1 cc. daily was added to the hay, oats,

and water diet (Chart 5). The oil was given partly for the same reason that the egg was added, but also on account of its particular therapeutic efficacy in preventing or curing rickets. It would seem that this diet constitutes an excellent standard dietary for experimental scurvy. It comprises the various necessary food elements, is laxative in character, is antirachitic, and in no degree antiscorbutic. It may be added that these results with cod liver

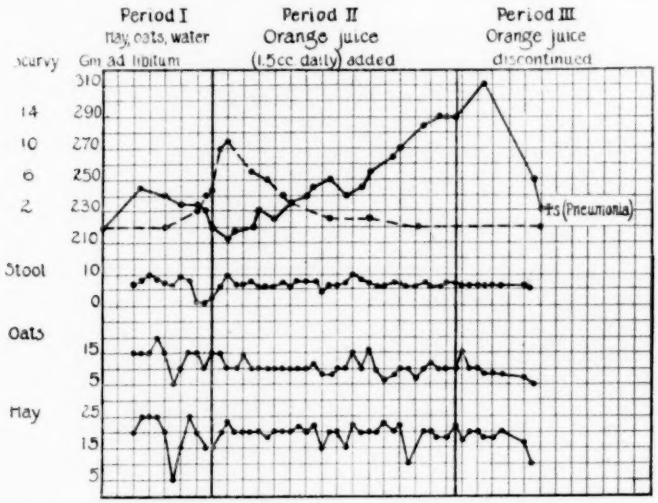

CHART 6. Shows a period with the development of scurvy, and one where it was cured by giving orange juice. It will be noted that there was no marked constipation while the animal had scurvy. During a period of a few days the amount of stool was scanty corresponding to the decreased intake of oats and hay on the days preceding.

oil and with egg merely tend to confirm similar results which have been reported by us in infantile scurvy.

In these animals which were receiving cod liver oil, we paid particular attention to the state of the bowels, in view of the opinion of McCollum that, "Scurvy in the guinea pig is the result of the retention of feces" (6). The stool was soft and somewhat oily. Chart 6 illustrates an experiment which was devised for the purpose of ascertaining the relation of constipation to scurvy. The intakes of oats and hay were noted and the droppings were

weighed daily. This experiment convinced us still further that constipation did not play an essential part either in the onset or in the cure of scurvy, and that orange juice does not owe its potency to its laxative effect. A large number of guinea pigs were then given liquid petrolatum (Squibb) daily in addition to the diet of hay, oats, and water. However, although this occasioned marked looseness of the bowels, indeed to such a degree that at times it had to be reduced in quantity, it afforded no protection against scurvy (Chart 7). The lack of result which we obtained from the addition of cod liver oil and of liquid petrolatum is probably due to the fact that the guinea pigs which develop scurvy on this diet are not constipated and do not manifest impaction of

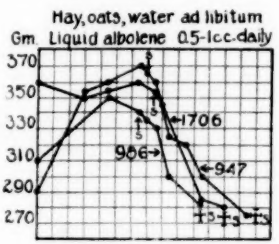

Chart 7. An attempt to prevent the occurrence of scurvy by giving liquid petrolatum (Squibb) daily. It will be noted, however, that this medication was unable to delay the onset of symptoms or the death of the animals.

the cecum after death. In the hundreds of autopsies carried out on guinea pigs which had died of scurvy, we paid particular attention to the contents of the intestine, and more particularly of the cecum, in this regard. In no instance have we encountered impacted feces in the cecum. Its contents have been pasty or semifluid, and in most cases it has contained gas. We have, however, noted massive feces, of the consistency of putty, in the cecum of animals fed on oats and milk. But as this dietary does not enter into this series of experiments, we shall postpone discussion of the condition until a future time. Although laxatives absolutely failed to prevent or relieve the the scurvy in our animals, McCollum has shown in definite instances that the guinea pigs with which he experimented were greatly benefited by liquid petrolatum.

It is difficult to harmonize these contradictory results. As we have mentioned above, it is impossible to interpret them in view of the fact that the dietaries were dissimilar. Furthermore, we cannot be certain that we are dealing with the same pathological condition until all results are controlled by histologic examinations. When studies are carried out in which these factors of the experiments agree, it seems certain that the marked differences of opinion regarding the pathogenesis of experimental scurvy will disappear and be fully explained.

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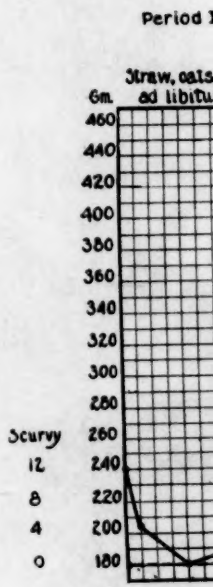

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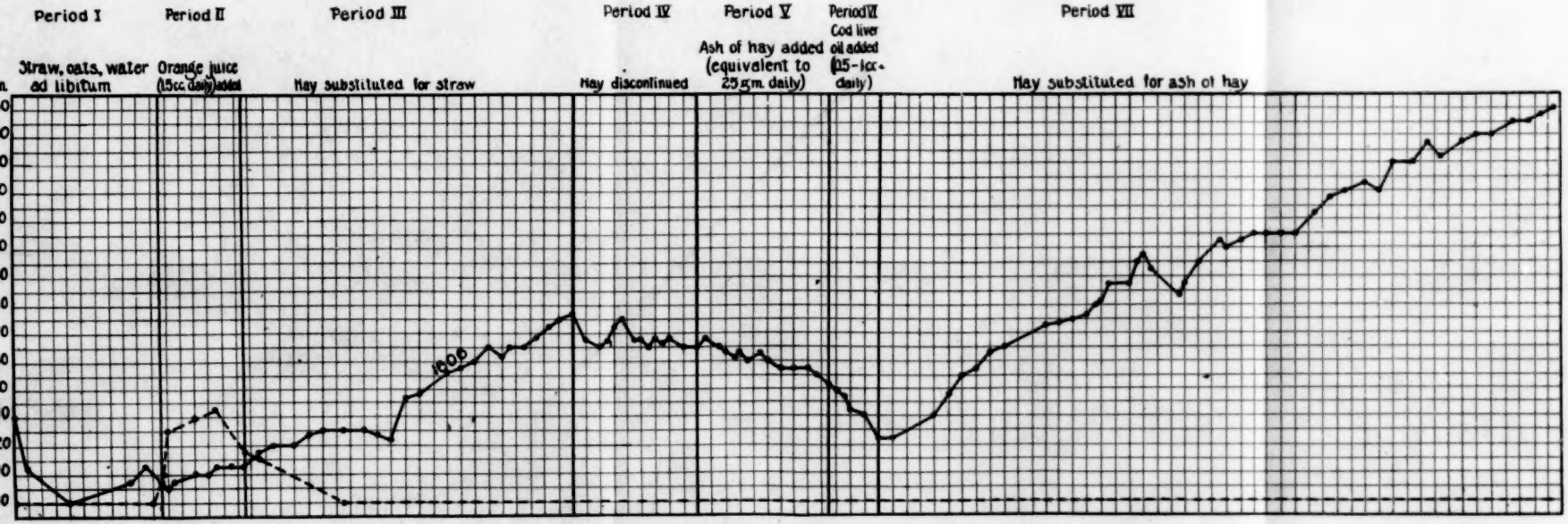

CHART 8. An experiment with seven feeding periods. Scurvy developed after the first, and was cured as usual by orange juice during the second. The third and fourth show that an animal can gain on a diet of hay, oats, water, and orange juice, but not on this diet minus the hay. The fifth and sixth periods show that this lack of gain is not due to the absence of the salts of the hay, nor to the absence of the fat-soluble vitamine. In view of the marked gain in weight during the last period where the only change was a substitution of the hay for its ash, we must believe that roughage was an important factor.

THE SCURVY OF GUINEA PIGS.*

II. EXPERIMENTS ON THE EFFECT OF THE ADDITION OF FRUITS AND VEGETABLES TO THE DIETARY.

By ALFRED F. HESS and LESTER J. UNGER.

(From the Bureau of Laboratories, Department of Health, New York.)

(Received for publication, June 25, 1918.)

In a previous communication (1) we have reported on scurvy brought about in young guinea pigs by a diet of oats, hay, and water *ad libitum*. As far as the occurrence of this disorder was concerned we noted no difference whether this simple dietary was employed, whether it was supplemented by the addition of cod liver oil to supply fat and fat-soluble vitamine, or whether the white and yolk of egg were added, enriching the diet with water-soluble vitamine, fat-soluble vitamine, fat, and protein. The signs of scurvy appeared regularly at the same period on the simple as on the more complete ration. In the preceding paper the gross pathologic changes noted at autopsy were checked by careful microscopic examinations of the bones, in order to be certain that the lesions were truly scorbutic. This precaution was considered essential in view of our encountering cases which were regarded as scurvy at autopsy but which histologically resembled rickets. A similar control of the pathologic anatomic changes was carried out in the present investigation.

This study concerns itself with the effect of augmenting the dietary with fruits and vegetables, and more particularly with the alteration in the antiscorbutic potency of these foodstuffs occasioned by heating, drying, and aging. This question of the exact food value of dried fruits and vegetables is important from a nutritional point of view, and is of especial significance at this time when dehydrated foods are being employed to an increasingly large extent both among the civil population and in army rations.

* A preliminary report of this work was presented before the Society for Experimental Biology and Medicine, May 15, 1918.

It is therefore highly desirable that we should have as complete knowledge as possible of their food value in the dried state.

As orange juice is the prototype of an antiscorbutic, it seemed highly desirable to investigate whether aging affected to any degree its antiscorbutic value. Accordingly three guinea pigs, which had developed scurvy on the regular ration, were fed 3 cc. daily of orange juice which had been kept in the refrigerator for over 3 months. As will be noted from Chart 1 this addition cured the scurvy in the two pigs which lived long enough to permit us to

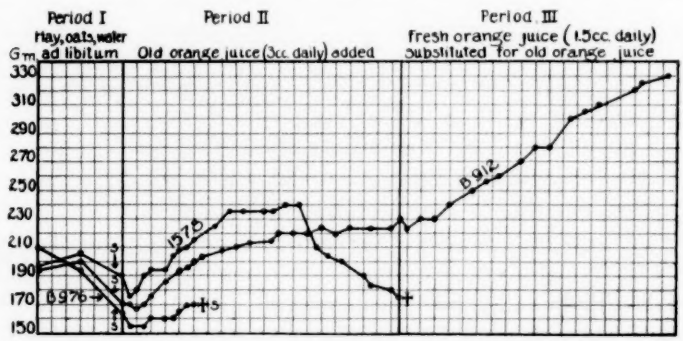

CHART 1. Hay, oats, and water *ad libitum* for first period during which time all animals developed scurvy. During the second period 3 cc. of old orange juice were added; this had been kept in the refrigerator for over 3 months. As a result two animals lost all signs of scurvy but the improvement was slow and they thrived only moderately well; the third guinea pig (No. B 976) died 10 days later and showed lesions of scurvy.

During the third period 1.5 cc. of fresh orange juice were substituted for the 3 cc. of the old preparation in the case of Guinea Pig B 912. The result was a sharp and continued gain in weight.

judge of the result. It seemed, however, to have lost some of its potency as the result of aging, for when fresh orange juice, to only half the amount, was substituted in the case of the dietary of one animal, there was a sharp rise in weight and an improvement in general condition. We may add that a similar clinical result was noted when guinea pigs were given orange juice which had been subject to heat. Animals fed juice which had been autoclaved at a temperature of 110° under 10 to 15 pounds pressure for a period of 45 minutes, did not thrive so well as those which were

fed the unheated juice. In both experiments, however, the signs of scurvy disappeared.

The active factor in orange juice can be extracted by means of 95 per cent alcohol. Chart 2 illustrates an experiment in which scurvy was cured by adding a small amount of such an extract to

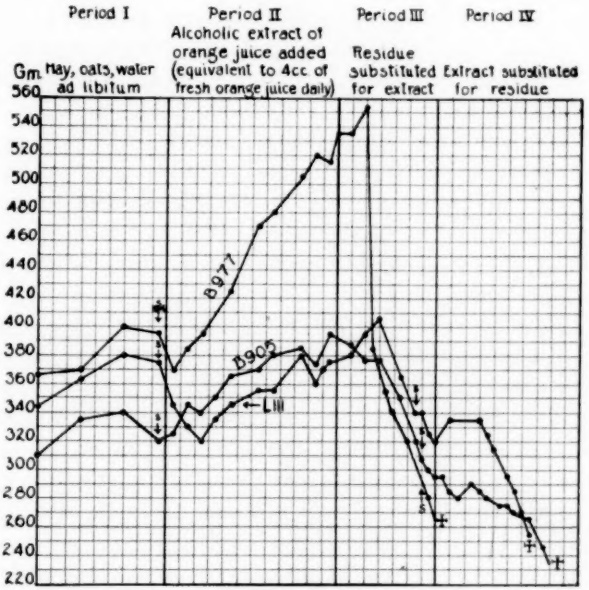

CHART 2. During Period I all guinea pigs developed scurvy on a diet of hay, oats, and water *ad libitum*. These signs all disappeared during the second period when alcoholic extract of orange juice equivalent to 4 cc. of the juice was given daily. During the third period the residue of this extract was substituted in the same dosage, with the result that scurvy reappeared and there was marked loss of weight. A short fourth period was begun wherein the extract was given in place of the residue. However, the guinea pigs died, although the signs of scurvy were ameliorated.

the dietary, and where it redeveloped when the residue was substituted for the extract. The extract was obtained as follows: 300 cc. of orange juice were evaporated at room temperature by means of a fan to about 40 cc. To this fluid 800 cc. of 95 per cent alcohol were added. This was allowed to extract at room temperature

for 7 days, being shaken frequently every day. It was decanted and once more evaporated down to about 40 cc., and then distilled water was added so that its volume again equalled 300 cc. (that of the original orange juice). The residue, which was of a gummy consistence, was taken up with distilled water so that it also corresponded with the original volume of the fruit juice. The water content of this extract varied somewhat in different preparations, but contained on an average 10 to 12 per cent of water. Its effect both on the scurvy and on the weight of the guinea pigs was striking when compared to the residue (Chart 2).

As it is not possible to define the nature of the antiscorbutic agent, it seems preferable, for the present, to term it "an accessory food factor." That its effect is not that of a mere laxative has been brought out in our previous paper (1) and is fortified by the negative results (which will be published elsewhere) obtained with laxatives in human scurvy. We may add that its remarkable therapeutic effect is not due to the mere salts frequently associated with it. In many instances we have attempted to cure the scurvy of guinea pigs by means of an addition of 1.5 cc. of "artificial orange juice" made according to the formula of McCollum and Pitz (2). This is composed of the various salts, citric acid, and sucrose in the proportions in which they are found in the natural juice. However, in every case it failed to show any curative properties. As we have mentioned in the preliminary communication, this same negative therapeutic effect was met with in the treatment of two cases of infantile scurvy treated for a period of about 3 weeks with appropriate amounts of this preparation. The most reasonable interpretation of the success obtained by others with "artificial orange juice" would seem to be that the guinea pigs which were benefited were suffering from some disorder not comparable with human scurvy.

A few years ago one of us reported the observation that the peel of the orange contains the antiscorbutic factor (3). This was of interest from a practical standpoint as it suggested the use of a waste product in place of or in addition to orange juice in connection with infant feeding. It seemed of interest not only to test further the value of orange peel in this respect, but to ascertain whether it would retain its antiscorbutic virtue in spite of drying (Chart 3). The peels which were used for this test were

kept in a burlap bag in an ordinary closet for a period of 3 months. By this time they had become dry and brittle. Their composition was as follows: total ether extract, 1.77 per cent; non-volatile ether extract at 100°, 1.58; volatile ether extract, 0.10; moisture 10.01 per cent. They were ground up before using, and 0.5 gm. of this substance was fed daily by means of a pipette, after thoroughly moistening with water, to a number of guinea pigs. Although all of these animals developed scurvy,

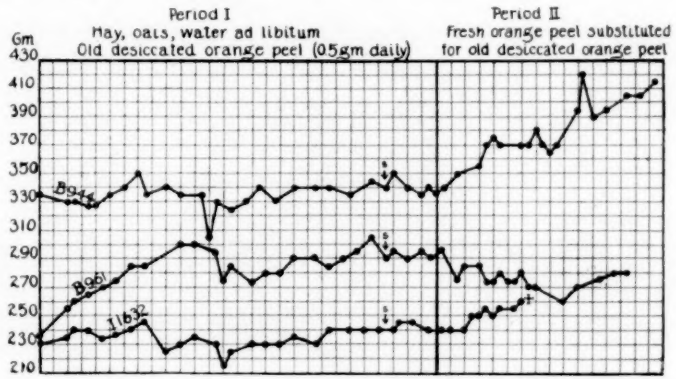

CHART 3. Hay, oats, water *ad libitum* and desiccated orange peel (0.5 gm. daily). This peel was about 3 months old, and had been dried at room temperature. It was powdered each day and given with a pipette.

It will be noted that it prolonged the life of the animals markedly. It also greatly delayed the onset of scurvy. After a period of 56 days an equivalent amount of fresh orange peel was substituted. In the case of Guinea Pig B 944 there was a prompt gain of weight, and a disappearance of the scorbutic signs. In the case of the two other animals the scurvy was cured in one (No. B 961) and was improved in the other.

they did not evince signs of this disorder for 50 days, proving that the peel still retained considerable of its antiscorbutic power. Doubtless had a larger dosage been employed the development of symptoms would have been postponed still further. This result shows that under certain physical or chemical conditions, which deserve closer study, the accessory food factor is not destroyed for a considerable period.

The effect of an addition of prunes to the oats, hay, and water dietary was tested. This fruit was singled out as it is used so

extensively in infant feeding, and it is, therefore, important for physicians to know whether it can replace the orange juice, which is commonly prescribed when raw milk is not fed. The prunes were soaked in a small amount of water, crushed in a mortar, and then fed. Chart 4 shows the failure of this foodstuff which is seen to have retained almost none of its antiscorbutic power. It is interesting to compare the result with that obtained with orange peel, of which only about 5 per cent of the quantity was given.

The second part of this study was designed to test the efficacy of dehydrating vegetables. Some years ago Holst and Frölich (4)

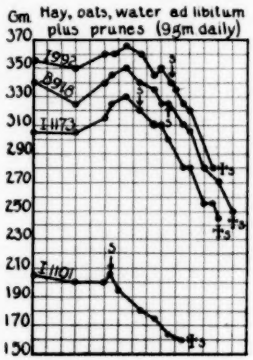

CHART 4. Hay, oats, water *ad libitum* plus 9 gm. of prunes. The juice of the prunes was given by means of a pipette, and the pulp was fed. It will be seen that this dry fruit had apparently no effect on the onset or severity of the scurvy.

showed by experiment that dried vegetables had lost their antiscorbutic power. This had been the sad experience in our army during the civil war (5) and in other armies previous to this time. The experiments of Holst and his coworkers have been repeated and amplified recently at the Lister Institute by Chick and Hume (6), who came to the same conclusions. Nevertheless there is widespread propaganda claiming that "dehydrated vegetables and fruits differ from fresh vegetables in only one respect, namely, in that they have had the moisture taken out" and again that "the trouble is that we like our vegetables fresh." We learn furthermore that in all probability in the near future

dehydrated vegetables will become a staple in our army rations. In view of these various activities, the subject assumes added importance at the present time.

The very fact that guinea pigs promptly develop scurvy on a diet which includes an unlimited supply of hay, in other words sun-dried grass, is strong experimental evidence against the retention of antiscorbutic potency after drying.

Charts 5, 6, and 7 portray the results of the addition to the regular dietary of three different types of dehydrated vegetables. In the first test dehydrated carrots, bought in the open market, were fed. In all these experiments the vegetable was mixed with

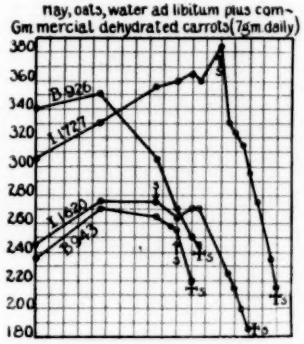

CHART 5. Hay, oats, water *ad libitum* plus 7 gm. of dehydrated carrots purchased in the open market. All the animals died with marked signs of scurvy.

as much water as it would absorb, precaution being observed not to add a surplus and thus wash out any of the salts. For the vegetables made use of in the two other experiments, a grinder was employed so that they would more readily take up moisture. It was found that this procedure rendered them more palatable to the animals. In all cases careful note was made of the amount of vegetable consumed. In general it may be stated that the carrots employed in Experiment 6 were not well taken, but that in Nos. 5 and 7 they were consumed in the amounts provided, excepting towards the end when the animals were weak and sick. The "commercial" dehydrated carrots possessed practically no scurvy-protecting power. It is not possible to state the age of this

preparation, or the degree of heat to which it had been subjected. The second lot tested (Chart 6) had been prepared 7 months previously by means of drying at room temperature with the aid of electric fans. It had meanwhile been carefully preserved in waxed cartons. These carrots likewise failed to ward off scurvy. When, however, fresh carrots were substituted all scorbutic signs rapidly disappeared and there was a prompt change in the general condition. The last experiment of this series (Chart 7) was the most conclusive as the carrots were excellent in appearance and flavor.[1] They had been dehydrated about 3 to 5 weeks before the

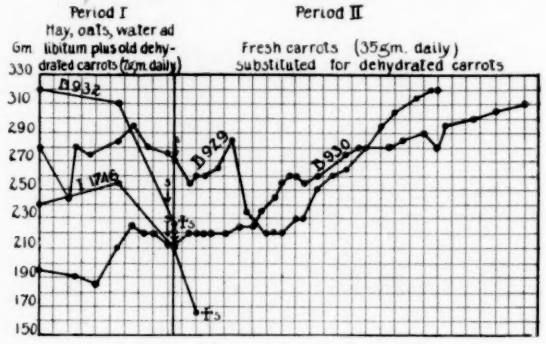

CHART 6. Hay, oats, water *ad libitum* plus 7 gm. of dehydrated carrots which had been dried about 7 months previously at room temperature. All animals developed scurvy. In the dietary of two an equivalent amount of fresh carrots was substituted, with the result that these two recovered, losing all signs of scurvy, whereas the other two died.

test, and meanwhile kept in well sealed paraffined paper bags. They were unable, however, to prevent or even notably to defer the onset of scurvy, and showed a sharp contrast in this respect to the potency of the fresh carrots which replaced them.

These experiments are not to be interpreted as showing that dehydrated vegetables are not a valuable foodstuff. On the contrary, in view of the fact that they are capable of furnishing a large amount of nutriment to the diet and their small bulk and ready preservation, they deserve commendation. They are not, how-

[1] These vegetables were furnished by the Mrs. Oliver Harriman Food Research Laboratory of New York.

ever, the equivalent of the fresh vegetables, and unless this distinction is clearly realized it will result in nutritional disorder. It is quite possible that the process of dehydration and of subsequent storing can be so improved as to obviate the loss of this food accessory. The method includes a number of factors, among which the degree of heat employed in dehydration seems less important than the rapidity of the process. The temperature and state of moisture at which the vegetables are stored have also an

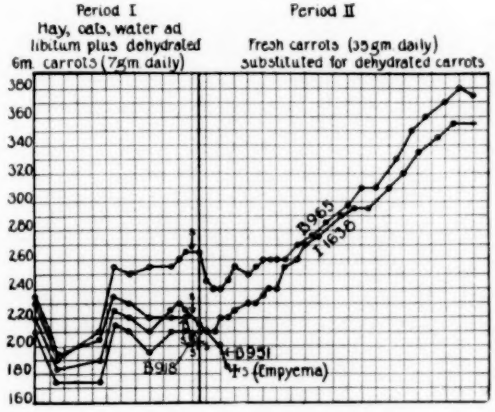

CHART 7. Hay, oats, water *ad libitum*, and 7 gm. of dehydrated carrots dried at a room temperature of about 130°F. about 1 month previously. All animals developed scurvy. The marked gain in weight when an equivalent amount of fresh carrots was substituted for the dehydrated vegetable corresponded to the disappearance of scorbutic signs. Guinea Pig B 951 did not receive fresh carrots, as it was evidently ailing at the time the second period began.

important bearing on their deterioration. Finally, it is probable that certain vegetables will be found to withstand dehydrating more successfully than others. It would seem that the onion and the cabbage possess the highest degree of antiscorbutic power, so that they might be expected to retain the greatest measure of this quality after proper drying and storing.

In conclusion we may add that these experiments with guinea pig scurvy coincide with our experience in the treatment of infantile scurvy. Indeed, unless the results of animal investigation can

be brought into harmony with clinical observation—particularly in regard to the efficacy of therapeutic agents—they must be looked upon as doubtful and inconclusive.

SUMMARY.

Orange juice which had been allowed to age for some months in the refrigerator was found to have lost some of its antiscorbutic power. This factor may be extracted from orange juice by means of 95 per cent alcohol, and is entirely absent in the residue.

"Artificial orange juice" prepared according to the formula of McCollum and Pitz failed to protect guinea pigs from or to cure them of scurvy. It was also found to lack antiscorbutic power in infantile scurvy.

Orange peel possesses marked antiscorbutic potency, and withstands desiccation remarkably well, retaining considerable of this power after being dried for 3 months. Prunes possess practically no value as a preventive against scurvy.

Dehydrated vegetables were found to contain little or no antiscorbutic virtue. This experience coincides with that encountered in relation to human scurvy. As this food is of decided nutritive value efforts should be instituted to improve the methods of dehydrating and of storing so as to obviate this deficiency.

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THE INFLUENCE OF ELECTROLYTES UPON THE OSMOTIC PRESSURE OF GELATIN SOLUTIONS.

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I.

It is generally stated in the literature of colloid chemistry that gelatin is influenced by both ions of a neutral salt, and that the influence of the anion of the salt is greater than that of the cation. The different anions are said to differ in their relative efficiency in a definite order, the now famous Hofmeister anion series. The writer has shown in a series of articles that all these statements are contrary to fact, and that the real facts are as follows: Common gelatin (*i.e.* gelatin near the point of neutrality) is affected only by the cations of a neutral salt, and not by the anions, univalent cations causing an increase in swelling, alcohol number, and viscosity, while bivalent cations cause no such change. The anion of the salt has no effect whatever.[1–5] The error into which the colloid chemists have fallen is due to the fact that they always investigated the effect of a neutral salt on a protein in the presence of the salt, while the writer took the precaution to wash the excess of salt away after it had time to act on the gelatin. In order to be able to do this he used gelatin in the form of a fine powder. A 1 per cent solution of the gelatin used for his experiments had a hydrogen ion concentration of 10^{-7} (pH = 7) (determined by Dr. Dernby). Since according to Michaelis[6] the

[1] Loeb, J., *J. Biol. Chem.*, 1917, xxxi, 343.
[2] Loeb, *J. Biol. Chem.*, 1918, xxxiii, 531.
[3] Loeb, *J. Biol. Chem.*, 1918, xxxiv, 77.
[4] Loeb, *J. Biol. Chem.*, 1918, xxxiv, 395.
[5] Loeb, *J. Biol. Chem.*, 1918, xxxiv, 489.
[6] Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914.

isoelectric point of gelatin is at $C_H = 2.10^{-5}$ (pH = 4.7) the hydrogen ion concentration of the solution was less than that of the isoelectric point. In a paper which will appear in the first number of *The Journal of General Physiology* the writer will show that on the alkaline side from the isoelectric point gelatin (and amphoteric electrolytes in general) can exist in an ionized condition only as anions, and hence is capable of combining only with the cation of a neutral salt, as we had actually found. When the hydrogen ion concentration of the solution is greater than that corresponding to the isoelectric point, the gelatin (and amphoteric electrolytes in general) can exist in an ionized condition only as cations and hence can combine only with the anion of a neutral salt. Hence the statement that always both ions of a neutral salt act on the gelatin, and that the effect of the anion prevails in the case of gelatin is contrary to fact as well as theory.

In a paper generally accepted by colloid chemists Lillie[7] has claimed that while salts depress the osmotic pressure of gelatin solutions, acids and bases have the opposite effect; and he also expresses the general belief that both ions of a salt are effective and that the relative depressing effect of the anions of neutral salts follows the order of the Hofmeister anion series.

Lillie's conclusions are as follows:

"Acid and alkali increase the osmotic pressure of gelatin solutions; in general these substances affect the osmotic pressure of gelatin solutions in the same manner as they do the rate of swelling of solid gelatin plates immersed in water.

"Addition of salts depresses the osmotic pressure of both colloids [gelatin and albumin]; the degree of depression is a function of the nature of both the anion and the cation of the salt. It increases in the order: alkali metals < alkali earths < heavy metals (for cations); and CNS < I < Br < NO_3 < Cl < F < plurivalent anions—SO_4, tartrate, citrate, phosphate (for anions)."[8]

The statement that acid and alkali increase the osmotic pressure while salts depress it is as we shall show merely due to the fact that Lillie worked with low concentrations of acids and bases in which the repressing effect of the presence of the electrolyte is less noticeable; while he worked with high concentrations of

[7] Lillie, R. S., *Am. J. Physiol.*, 1907–08, xx, 127.

[8] Lillie, *Am. J. Physiol.*, 1907–08, xx, 169.

the neutral salts in which the repressing effect of the presence of the electrolyte was noticeable. Had he used stronger concentrations of alkalies and acids he would have noticed that the depressing effect which he observed in the case of salts is equally pronounced in the case of acids and bases. We shall show in this paper that the influence of electrolytes upon the osmotic pressure of gelatin runs parallel to the influence of the electrolytes upon the swelling, viscosity, and alcohol number of gelatin, demonstrated by our previous papers, inasmuch as only the cation of a neutral salt influences the osmotic pressure, while the anion is without influence upon common gelatin. We shall show, moreover, that while the salts with monovalent cation increase the osmotic pressure, the salts with bivalent cation have no such effect. The same difference exists between the influence of bases with univalent and bivalent cation and between monobasic and dibasic acids. In order to show this we have to wash away the excess of the electrolyte after it has had time to act on the gelatin.

The osmotic pressure was determined by the simple method devised by Lillie.[7] Small bags in the shape of Erlenmeyer flasks were made of collodion and the neck of each bag was fitted into a perforated rubber stopper and made tight with the aid of rubber bands. A vertical glass tube of about 2 mm. diameter went through the hole of the stopper into the gelatin solution which at the beginning filled the bag and the lower end of the glass tube to about 20 mm. All air bubbles were carefully removed from the bag. The latter was then put into a large beaker containing about 250 cc. of distilled water. The beakers were in a water bath kept at a temperature near 20°C. The final equilibrium of the column of liquid in the glass tube was reached over night and remained constant for the next 24 hours. Our final measurements were usually made after about 16 hours.

It was found that our collodion bags were easily permeable for salts like NaCl, but were practically impermeable for gelatin. When a bag was filled with a M/2 NaCl solution and put into distilled water, the liquid in the bag or the glass tube serving as manometer rose during the first half hour to a height of about 70 mm. and then fell rapidly. In a few hours the osmotic pressure of the solution was zero. When the same experiment was made

with a 1 per cent gelatin solution put into a beaker with distilled water, the pressure rose slowly to a height of about 80 mm. and remained there for several days—in fact as long as we continued the experiments. A test made of the distilled water surrounding the bag failed to give the biuret test after 16 hours.

Lillie's method may not be sufficiently accurate to satisfy the demands of the physicist,[9] but it is adequate to allow us to show that our former statements concerning the influence of neutral salts upon the physical properties of gelatin (swelling, viscosity, and resistance to precipitability by alcohol) hold also for the influence of neutral salts upon the osmotic pressure of gelatin solutions.

II.

On the basis of our previously published experiments we should expect that a treatment of powdered gelatin with a neutral salt with univalent cation should cause an increase in the osmotic pressure of the gelatin, after the excess of the salt solution is washed away, since the treatment of the gelatin with neutral salts should lead to the formation of metal gelatinates. The higher the concentration of the salt solution used, the greater the mass of metal gelatinate formed and the greater the increase in osmotic pressure to be expected. On the other hand, treatment of gelatin with neutral salts with bivalent metal should on the basis of our previously published experiments not cause any increase in osmotic pressure after the excess of salt has been washed away. Table I shows that these theoretical expectations are all fulfilled.

The method was the same as that used in the experiments on alcohol precipitability. 1 gm. of powdered gelatin going through 50 mesh sieve but not through one of 60 was put for 1 hour into 100 cc. of the solution of a neutral salt to bring about a chemical reaction between gelatin and salt. The powder was then put on a filter, the salt solution allowed to drain off, and the gelatin was perfused six times with H_2O to remove the last traces of salt

[9] Two factors in our present method seem to cause a variation in the results; first, possible differences in the permeability of the membranes, and second, differences in the degree of washing away the electrolytes in solution and, if the washing is excessive, in combination with the gelatin,

solution held in the capillary spaces or adhering to the surface of the granules. The gelatin was then heated for about 10 minutes in a water bath of 50°C. so as to liquefy the gelatin, and distilled water was added to make the solution 1 per cent. With this gelatin solution free from the salt with which the gelatin had been treated the influence of salts upon the osmotic pressure was determined.

TABLE I.

	Osmotic pressure in mm. of 1 per cent gelatin solution in distilled water, made from powdered gelatin treated for 1 hour with one of the following solutions and then perfused six times with 25 cc. H_2O to wash away the excess of salt solution.											
	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	M/4096	M/8192	H_2O
NaCl	134	122	123	107	99	93	81	80	77	77	71	74	77
NaBr	136	133	123	118	107	88	78	79	82	78	76	78	79
NaCNS		132		118	107	94	94	82	88	78	75	73	74
Na acetate	170	151	139	127	108	92	89	80	79	78	77	75	81
KCl	116	120	116	100	95	80	72	75	68				70
$LiNO_3$	121	120	104	102	91	84	81	73	73				73
NH_4Cl	145	138	137	127	105	94	86	80	83	81	77	77	77
Na_2SO_4	159	166	156	151	136	107	102	83	80				73
Na_2 tartrate	166	159	142	129	116	110	98	77	60	73	78		72
Na_2 succinate		170	163	144		132	112	91	83	86	81		84
Na_2 oxalate		100	91	88	90	94	90	88	81	78	82	81	
Na_2 malate		166	152	142	135	121	102	89	76	79	77	73	
$MgCl_2$		79	83	82	82	77	78	81	81	75	78	78	80
$CaCl_2$	77	76	82	76	84	84	83	80	81				80
$SrCl_2$	75	81	78	80	83	81	82	82	76				78
$BaCl_2$		69	70	73	76	76	75	80	82	81	81	80	82
$MgSO_4$	84	80	80	82	83	82	79	81	80	73	82	82	81

In looking at the results in Table I it is strikingly clear, first, that a treatment of the gelatin with a neutral salt with a bivalent metal, $MgCl_2$, $CaCl_2$, etc., leads to no increase in osmotic pressure, even if we go up to M/4 solutions. (We cannot go beyond this limit on account of the liquefying effect of the higher concentrations upon the gelatin and the difficulty of freeing the solution from the excess of salt.)

Second, it is obvious that $MgSO_4$ acts exactly as $MgCl_2$, thus indicating that the anion has no influence.

Third, it is obvious that the salts with univalent cation increase the osmotic pressure of the gelatin.

Fourth, it is obvious that the molecular concentration required to cause a perceptible rise (to 90 mm. or above) in the osmotic pressure is for salts of the type NaCl (univalent cation, univalent anion), higher than for salts of the type Na_2SO_4 (univalent cation and bivalent anion). For the former it is between M/128 and M/256, and for the latter between M/256 and M/512; indicating that the concentration of the cation alone (or practically exclusively) determines the effect of the salt regardless of the nature of the anion, though this latter result does not come out as strikingly as in the measurements of the precipitability of gelatin with alcohol or the limiting concentration for additional swelling.[2, 3] Within the limits of the imperfections of our present osmotic pressure determinations the results on osmotic pressure confirm our theory of the action of neutral salts upon proteins expressed in our former papers. The only exception noticed is the oxalate solution, which apparently caused a precipitate in the gelatin.

III.

Our theory furthermore demands that bases should influence the osmotic pressure of gelatin in a sense parallel to that of the corresponding neutral salts; namely, a previous treatment of powdered gelatin with NaOH and KOH should cause a considerable increase in osmotic pressure (after the free base is washed away) while $Ba(OH)_2$ and $Ca(OH)_2$ should cause no or at the best only a slight increase in osmotic pressure under the same conditions of experimentation. According to our theory bases act upon proteins of the type of gelatin (provided that pH > 4.7) like neutral salts, both causing the formation of metal proteinates, while the anion of the salt has apparently no influence upon the protein. A glance at Table II shows this expectation to be correct. 1 gm. of powdered gelatin was put for half an hour into the solutions of the different bases and then treated in a similar way as in the case with salts. The excess of alkalies was allowed to drain off and the gelatin was washed four times with

25 cc. of H_2O. A treatment of the gelatin with $Ca(OH)_2$ leads to no increase in the osmotic pressure of the solution, while a treatment with $Ba(OH)_2$ may lead to a slight increase after the excess of base has been washed away. On the other hand, treatment of the gelatin with NaOH and KOH leads to a considerable increase in the osmotic pressure after the excess of alkali has been washed away.

TABLE II.

	Osmotic pressure in mm. of 1 per cent gelatin solution in distilled water, made from powdered gelatin treated for 30 min. with 100 cc. of one of the following solutions and then perfused four times with 25 cc. H_2O to wash away the excess of salt solution.										
	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	M/4096	M/8192	H_2O
NaOH.....	172		165	129	115	102	98	90	88	90	76
KOH.....	154	155	147	120	98	96	95	88	87	79	77
$Ba(OH)_2$..		77	95	95	97	99	95	90	89	88	
$Ca(OH)_2$..			85	89	92	87					78

IV.

The fact that salts or bases with bivalent cation do not lead to an increase in the osmotic pressure (or the swelling, the viscosity, and resistance to alcohol precipitation) of proteins might be interpreted to mean, that such salts or bases do not combine with proteins. Such a view is, however, repudiated by the facts of antagonistic salt action, inasmuch as it can be shown that the addition of a comparatively small amount of a salt with bivalent cation inhibits the increase in osmotic pressure (and also the increase in swelling, viscosity, and resistance to alcohol precipitation)[1–4] caused by a treatment of the proteins with a salt or base with univalent cation after the excess of salt solution is washed away.

The method of our experiment was as follows: 1 gm. of powdered gelatin was put for 1 hour into 100 cc. M/8 NaCl containing 0, 1, 2, 4, 6, 8, 12, 16, and 32 cc. M/8 $CaCl_2$ or $BaCl_2$. After 1 hour the gelatin was put into a funnel, the salt solution was allowed to drain off, and the powder was then perfused six times with 25 cc. of H_2O to remove the last traces of the salt solution. After

this, 1 per cent solutions of these different gelatins in distilled water were made, and the osmotic pressures were determined. It was found that the addition of Ca or Ba diminishes the rise in osmotic pressure which is caused by the pure solution of NaCl, and this diminution is the greater the more Ca or Ba had been added. The addition of 1 or 2 cc. of $CaCl_2$ or $BaCl_2$ had already a noticeable effect (Table III).

This proves that the salts with bivalent cation prevent the increase of osmotic pressure caused by the salts with univalent cation. The same antagonism has already been demonstrated in the writer's previous papers for additional swelling, increase in viscosity, and resistance to alcohol precipitation. The reader will remember that in all these experiments the excess of salt solution was washed away after the salt had had time to act on the gela-

TABLE III.

	Osmotic pressure in mm. of 1 per cent gelatin solution in distilled water, made from powdered gelatin treated for 1 hour at 20° with 100 cc. of an M/8 NaCl solution containing various amounts of M/8 $CaCl_2$ or $BaCl_2$, and then perfused six times with 25 cc. H_2O to wash away excess of salt.									
100 cc. M/8 NaCl + cc. $CaCl_2$ or $BaCl_2$	0	1	2	4	6	8	12	16	24	32
NaCl + $CaCl_2$	131	129	120	107	106	99	94	90	85	84
NaCl + $BaCl_2$	134	125	111	98	110	29	97	83	86	74

tin. These experiments prove that the salts with bivalent cations react with gelatin.

According to our theory the same antagonism as between NaCl and $BaCl_2$ should exist between NaOH and $Ba(OH)_2$. This was found to be true.

Powdered gelatin was put into 100 cc. of M/32 NaOH (free from carbonate) containing 0, 1, 2, 4, 6, 12, 16, 24, and 32 cc. M/32 $Ba(OH)_2$. The mixture was contained in tightly stoppered bottles, and the powder remained in the mixture 30 minutes. After this the gelatin was put into funnels, the alkali solution allowed to drain off, and the gelatin was perfused four times with 25 cc. of H_2O to remove the last traces of the original solution from the capillary spaces between the granules. Table IV shows that the expected antagonism between NaOH and $Ba(OH)_2$ was

found, and in addition that the antagonistic action exists also for swelling and precipitation with alcohol.

These experiments are only intelligible on the assumption that the salts and bases with bivalent cation influence the protein in the same way and that, moreover, the bivalent cations inhibit the effect of the salts and bases with univalent cation.

TABLE IV.

	1 gm. powdered gelatin put for 30 min. into one of the following solutions and then perfused four times with 25 cc. H_2O.								
100 cc. M/32 NaOH + cc. M/32 $Ba(OH)_2$	0	1	2	4	8	12	16	24	32
Osmotic pressure in mm. of 1 per cent gelatin solution	185	147	148	147	133	126	115	109	105
Total swelling in mm. of height of cylindrical mass of gelatin	70	71	70	61	50.5	51	46	45	31
Cc. 95 per cent alcohol required to precipitate 5 cc. of 1 per cent gelatin solution	∞	∞	∞	∞	∞	14.9	11	10.4	7.1

V.

We will now show that if we do not remove the excess of solution of electrolyte with which the gelatin is treated, the effects of the electrolyte will be repressed and that this repression varies with the concentration and nature of the electrolyte. The measurements for the effect of electrolyte upon the osmotic pressure of gelatin will thus be the resultant of opposite influences of the electrolyte, the relative quantity of which is unknown in each case; and what appears to be the effect of the electrolyte upon the gelatin is in reality the difference between the unknown effect of the electrolyte upon the gelatin and the equally unknown repressing effect of the presence of the excess of electrolyte. Instead of arriving at the clear results which our method gives, we arrive at indefinite results which misled Hofmeister into the erroneous statement concerning the effect of anions upon the

swelling;[10] and the authority of Hofmeister obviously induced Lillie to look for a confirmation of Hofmeister's statement.

The experiments represented in Table V were made in a similar way as those of Lillie. A 1 per cent solution of gelatin was made up in the solution of the electrolyte whose effect was to be studied, and the same solution of the electrolyte was put into the beaker surrounding the collodion bag containing the 1 per cent gelatin solution. Since the membrane is entirely permeable

TABLE V.

	Osmotic pressure in mm. of 1 per cent gelatin solution in presence of electrolyte.													
	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	M/4096	M/8192	M/16384	H_2O
I.														
HNO_3	47	48	58	46	104	169	286	352	330	205	81	26		
NaOH			71	70	85	124	122	213	206	185	134	112		90
NaCl		37	33	38	35	41	42	63	74	83	90	84		88
II.														
H_2SO_4				39	48	62	90	121	93	140	103	34	46	81
$Ba(OH)_2$					60	49	50	71	102	107	115	110		
$BaCl_2$		35	32	30	31	33	33	32	38	42	66	73		
III.														
$NaNO_3$		32	34	28	38	39	50	61	62	81	78	91		87
LiBr		35	35	42	42	39	58	62	71	70	89	87		
Na acetate		43	43	45	Lost.	52	66	70	80	101	118	110		91
NaCNS			43		55	56	60		85	95	100	100		102
Na_2 tartrate			40	37	38	41	62	56	60		100	105	103	
Na_2SO_4		27	25	41	46	35	41	47	50	62	92	89		90

to crystalloids, the osmotic pressure of the gelatin solution can be measured in this way.

Group I, Table V, gives the osmotic pressure of a 1 per cent gelatin solution in the presence of a monobasic acid, HNO_3, a base with univalent cation, NaOH, and a neutral salt, NaCl. It is obvious that the presence of the base NaOH depresses the osmotic pressure below that of a non-treated gelatin solution as soon as the concentration of NaOH is M/64 or above; and the

[10] Hofmeister, F., *Arch. exp. Path. u. Pharmakol.*, 1891, xxviii, 210.

same is true as soon as the concentration of the acid is M/32 or above. According to Table I NaCl only begins to cause a marked increase in osmotic pressure in concentrations above M/128 or M/64, but the presence of the salt in these or higher concentrations prevents the manifestation of this increase.

Hence the behavior of gelatin in the presence of electrolytes conforms with the results and conclusions from our former experiments.

Furthermore, a glance at Group II, Table V, brings out a further confirmation of our theory; namely, that neutral salts with bivalent cation, $BaCl_2$, behave like bases with bivalent cation, $Ba(OH)_2$, and acids with bivalent anion, H_2SO_4, inasmuch as in all three cases the osmotic pressures run lower than the corresponding pressures in Group I, which was to be expected according to our previous publications.

Group III, containing different salts of Na, shows that there is no basis for assuming the validity of the Hofmeister series upon the osmotic pressure. The variations between the effects of sodium salts with different anions are within the limits of error of the method or due to differences in the repressing effect of the different salts present. Neither do Lillie's own observations support the validity of the Hofmeister series. He states:[11] "Individual variability is so strongly marked in colloidal solutions that little reliance can be placed on the order of action observed in a single series of determinations. . . . ." When we free the solution from an excess of salt, the results become clear and leave no doubt that the alleged anion effect of neutral salts upon gelatin does not exist.

SUMMARY.

1. It is generally stated in colloid chemistry that only acids and bases cause an increase in the swelling, the viscosity, and the osmotic pressure of hydrophilic colloids, while neutral salts of the type of NaCl have little or, according to Lillie, a directly opposite effect. The writer has found that neutral salts act exactly like acids and bases inasmuch as neutral salts and bases with univalent cation and acids with univalent anion

[11] Lillie, *Am. J. Physiol.*, 1907–08, xx, 152.

increase the swelling, the viscosity, and resistance to precipitation by alcohol of gelatin, while salts and bases with bivalent cation and acids with bivalent anion (with the exception of some organic acids) have no or only a slight effect. In this paper it is shown that the same is true for the influence of electrolytes upon the osmotic pressure of gelatin.

2. It was found by the writer that only the cation of neutral salts acts upon gelatin in neutral solutions or in solutions with pH > 4.7, while the anion of neutral salts does not influence the osmotic pressure and the other physical qualities of gelatin (swelling, viscosity, and alcohol precipitability).

3. These experiments contradict the statements generally made in colloid chemistry that the influence of neutral salts upon a protein is the algebraic sum of the opposite effects of the oppositely charged ions of the neutral salt. They also contradict the statement that common gelatin (at the point of neutrality) is influenced more strongly by the anion than by the cation of a neutral salt.

4. The difference between our results and those of the older experimenters is due to the fact that in the older experiments the effects of neutral salts upon gelatin were always measured in the presence of the salts. We were able to show that the presence of the salt represses the manifestation of its effects upon the gelatin and that it is therefore necessary to remove the excess of the salt after it has had time to act on the protein.

5. Our results are in agreement with a theory of the behavior of amphoteric electrolytes, which will appear in *The Journal of General Physiology;* namely, that on the more acid side of their isoelectric point amphoteric electrolytes can exist, when ionized, only in the form of cations, while on the less acid side of their isoelectric point they can exist, when ionized, only as anions. In the former case the ampholyte can combine only with the anion, in the latter only with the cation of a neutral salt. Since gelatin has its isoelectric point at pH = 4.7, while the gelatin used by us had a pH = 7, such gelatin could react only with the cation of neutral salts.

A SIMPLE APPLICATION OF THE VOLHARD PRINCIPLE FOR BLOOD PLASMA CHLORIDES.

By W. C. RAPPLEYE.

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(Received for publication, July 17, 1918.)

In an attempt to secure a convenient, rapid, and accurate method of determining the chloride content of blood plasma, the following application of the Volhard principle was found to be satisfactory. This principle is the precipitation of the chlorides by a standard solution of silver nitrate and the subsequent titration of the excess silver nitrate by potassium sulfocyanate, using iron-ammonium-alum as an indicator. The end-point is the first permanent reddish tinge due to the formation of ferricyanate when all the silver nitrate is combined with the titrating solution of potassium sulfocyanate. Knowing the amount of silver nitrate which has reacted with the halide present and the value of the silver nitrate solution in terms of sodium chloride, allows one to ascertain the amount of sodium chloride present in the original fluid examined.

In making determinations of substances in the blood, a method for such determination must be sufficiently accurate to allow the use of small samples. The objection to the usual Volhard procedures is that the solutions, especially the back titrating solution, are too strong. Each unit of measurement is equivalent to so much sodium chloride that a variation in back titration of a single drop (0.05 cc.) introduces a considerable error in the determination of the sodium chloride in a small sample, say 2 cc.

The following solutions were made up.

Solution I.

Silver nitrate	7.2653	gm.
Nitric acid (concentrated)	250.0	cc.
Saturated solution of iron-ammonium-alum	50.0	"
Distilled water to make	1,000.0	"

Solution II.

Potassium sulfocyanate in distilled water of such strength that 25 cc. is exactly equivalent to 5 cc. of the silver nitrate solution. Each cc. of the silver nitrate solution is equivalent to 2.5 mg. of sodium chloride and each cc. of the potassium sulfocyanate solution is equivalent to 0.5 mg. of sodium chloride.

Procedure.

1. Place 2 cc. of citrated plasma (oxalated plasma is not satisfactory) in a 50 cc. volumetric flask containing 30 cc. of distilled water.
2. Add 10 cc. of Solution I and make to mark.
3. Allow the mixture to stand 5 to 10 minutes.
4. Filter through a dry filter paper free from chlorides (or separate precipitate by centrifugalization).
5. Take 25 cc. of the filtrate and back titrate with Solution II, using a white background.
6. Subtract the number of cc. of Solution II from 25 and multiply by 50 to obtain the number of mg. of sodium chloride per 100 cc. of blood plasma.

Accuracy.

It has been mentioned that each cc. of the back titrating solution is equivalent to 0.5 mg. of sodium chloride, hence a variation of 0.05 cc. (about one drop) in back titration represents a variation in the final figure of 2.5 mg. of sodium chloride per 100 cc. of plasma. The end-point can be obtained consistently to within two drops, and, since the determinations are made on fluid containing about 575 to 600 mg. per 100 cc., the error is less than 1 per cent. The potassium iodide solution used for back titration in the McLean and Van Slyke method is of such a strength that a variation of 0.05 cc. (about one drop) in the back titration introduces a variation in the final figure of 6.25 mg. per 100 cc. of plasma.

To test the accuracy of the method, determinations were made of the chloride content of the plasma of a number of individuals by this method, and then salt to the value of 100 mg. per 100 cc. was added and the values were again determined. The fig-

ures are given below. The theoretical values (in parentheses) after the addition of salt assume the original determinations (at the left) to be correct.

Plasma.	Determined.	Added.	Recovered.	Theoretical value.
	mg.	*mg.*	*mg.*	*mg.*
I	600.0	100.0	700.0	(700.0)
	595.0	100.0	697.5	(695.0)
II	587.5	100.0	687.5	(687.5)
	585.0	100.0	680.0	(685.0)
III	595.0	100.0	695.0	(695.0)
	600.0	100.0	695.0	(700.0)
IV	617.5	100.0	715.0	(717.5)
V	612.5	100.0	715.0	(712.5)
VI	595.0	100.0	692.5	(695.0)
VII*	657.5	100.0	752.5	(757.5)
	655.0	100.0	755.0	(755.0)
VIII	642.5	100.0	745.0	(742.5)
IX	595.0	100.0	690.0	(695.0)
X	617.5	100.0	712.5	(717.5)

* Ascitic fluid.

To check the method further, parallel determinations were done on samples of plasma by this method and by the McLean and Van Slyke method. A comparison of the values obtained follows:

Plasma.	McLean and Van Slyke Method.	Author's Method.
	mg.	*mg.*
I	593.75	590.0
	593.75	590.0
II	600.0	605.0
III	587.5	592.5
IV	625.0	617.5
V	606.25	602.5
	606.25	605.0
VI	587.5	585.0
	593.75	587.5
VII	631.25	637.5
VIII	593.5	587.5

When possible to secure enough fluid to permit the use of larger samples (pleuritic or ascitic fluid, ample blood, etc.), the plan of using 5 cc. is followed. In such instances, place 5 cc. in 100 cc. flask containing distilled water, use 25 cc. of Solution I and back titrate as before, with 25 cc. of the filtrate. The figuring is a little different, owing to the different relationship of the standard solutions. Instead of subtracting the sulfocyanate figure from 25 and multiplying by 50, to secure the number of mg. of sodium chloride in 100 cc. of the fluid examined, subtract the back titration figure from 31.25 and multiply the result by 40.

Another modification can be made by using a potassium sulfocyanate solution, each cc. of which is equivalent to 1.0 mg. of sodium chloride. By making this solution stronger, a clearer definition of the end-point is obtained but, of course, it is not as delicate a back titrating solution as the one mentioned above.

THE DETERMINATION OF CREATININE AND CREATINE IN BLOOD.

By W. DENIS.

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(Received for publication, July 22, 1918.)

The method of Folin[1] for the determination of creatinine and creatine in blood, which has gained extensive use, particularly in connection with clinical material, has, during the past 2 years been the subject of critical study by Wilson and Plass,[2] by Hunter and Campbell,[3] and more recently by Greenwald and McGuire.[4] The conclusions reached by these three groups of investigators have been in the main identical; *i.e.*, that the results obtained for preformed creatinine and for total creatinine (creatine plus creatinine) are much too high; in fact, according to Hunter and Campbell, the figures for creatine in whole blood may be "four times as high as the amount [of this substance] actually present."

A method for the determination of creatine and creatinine in blood has been described by Wilson and Plass, which according to these investigators gives more accurate results than the Folin procedure. This method consists essentially in the precipitation of the blood proteins with dilute acetic acid and aluminium hydroxide, determination of the preformed creatinine in the filtrate (after appropriate concentration) and total creatinine in the same after heating with hydrochloric acid.

Greenwald and McGuire have more recently described two methods for the determination of creatine and creatinine in blood. In one, precipitation is accomplished by means of dilute acetic acid and "dialyzed iron," in the second procedure trichloracetic acid is used to remove the proteins. In addition these investigators

[1] Folin, O., *J. Biol. Chem.*, 1914, xvii, 475.

[2] Wilson, D. W., and Plass, E. D., *J. Biol. Chem.*, 1917, xxix, 413.

[3] Hunter, A., and Campbell, W. R., *J. Biol. Chem.*, 1917, xxxii, 195.

[4] Greenwald, I., and McGuire, G., *J. Biol. Chem.*, 1918, xxxiv, 103.

have introduced the use of kaolin which they have found to remove creatinine quantitatively from solution but to be without effect on creatine.

Until a sufficient time has elapsed to allow these new processes to be given a thorough trial, it would perhaps appear superfluous to publish further methods or modifications. It has, however, seemed worth while to describe a procedure for the determination of creatine and creatinine in blood which has been used in this laboratory for some time, because, while it is in all probability in no way superior to the trichloroacetic acid method of Greenwald and McGuire, it is considerably less time consuming than the methods which involve coagulation with boiling acetic acid, and is, we find, extremely convenient to use where it is desirable to determine both non-protein nitrogen and creatinine in the same sample of blood.

The method of precipitation is essentially the same as that described by Folin and Denis[5] for the determination of non-protein nitrogen in blood by direct Nesslerization.

Blood is collected in bottles containing powdered potassium oxalate (0.10 gm. of oxalate to 10 to 15 cc. of blood). 10 cc. of blood or plasma are pipetted into a 50 cc. volumetric flask containing an approximately equal quantity of distilled water, and 6 cc. of a 20 per cent solution of *m*-phosphoric acid are then added. The flask is shaken, allowed to stand for at least 1 hour, then made up to volume with water, and the contents are filtered. Two 10 cc. portions of the filtrate are measured out, one into a 25 cc. volumetric flask, the other into a similar flask of 50 cc. capacity.

For the determination of preformed creatinine add to the first portion (contained in the 25 cc. flask) 10 cc. of saturated picric acid solution, and 2 cc. of 10 per cent sodium hydroxide solution; allow the flask to stand for 10 minutes, make up to volume, and read against an appropriate standard.

For normal blood, standards containing 0.01 and 0.02 mg. of creatinine are usually all that are required. For work on pathological material, however, it has been found necessary to use standards containing 0.03, 0.04, 0.05, 0.08, and 0.10 mg.

It has been found most convenient to retain the principle described in connection with Folin's original method of making up

[5] Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 491.

these stock standard solutions with saturated picric acid,[6] as in this media the creatinine appears to keep indefinitely; in all, seven standard solutions are provided, made up in saturated picric acid to contain from 0.01 to 0.10 mg. of creatinine in 10 cc.

To prepare a standard for any given determination, place 10 cc. of one of the above standard solutions in a 25 cc. volumetric flask, add 10 cc. of distilled water and 1.5 cc. of 10 per cent sodium hydroxide solution, let stand 10 minutes, then make up to volume with distilled water.

To determine total creatinine (creatine plus creatinine) heat 10 cc. of the original *m*-phosphoric acid filtrate for half an hour in an autoclave at 120°C. or add about 75 cc. of distilled water and heat to boiling on a hot plate for 3 hours. When cool, add to the solution (which should have a volume of approximately 10 cc.) 10 cc. of saturated picric acid solution, 2 cc. of 10 per cent sodium hydroxide, and let stand for 10 minutes; then make up to volume (50 cc.) and read against an appropriate standard. The standards most useful in work on normal material are those containing 0.08 or 0.10 mg. of creatine; for pathological blood standards containing 0.15, 0.20, and 0.30 mg. are sometimes necessary.

The standards are prepared in the same general manner as described for the determination of preformed creatinine; *i.e.*, 10 cc. of the appropriate picric acid standard solution, 10 cc. of distilled water, and 1.5 cc. of 10 per cent sodium hydroxide.

Blood filtrates prepared by means of *m*-phosphoric acid, when treated with alkali and picric acid as described above, reach their maximum color in 8 minutes, the continued development of color after 10 minutes standing which is observed in the blood filtrates obtained by means of the picric acid precipitation being entirely absent.

In Table I are presented a few of the results obtained by the use of the above method. As will be seen the figures obtained for preformed creatinine are slightly lower than those secured by

[6] All the picric acid used in this work had been purified according to the method of Folin and Doisy (*J. Biol. Chem.*, 1916–17, xxviii, 349). Our "saturated" picric acid solutions had been made up to contain 1.2 per cent picric acid.

A Duboscq colorimeter was used for the color comparisons, the standard solution being set at 20 mm.

following Folin's method, while in the case of the results on total creatinine the discrepancy becomes relatively large. That these low results are not due to mechanical losses of creatinine or creatine or to chemical decomposition of these bodies by the reagents used is proved by the fact that I have repeatedly carried small quantities of pure solutions through the process as above out-

TABLE I.

Creatinine and Creatine in the Blood of Hospital Patients. Mg. per 100 Cc. of Blood.

No.	Diagnosis.	Folin method.		Proposed method.	
		Preformed creatine.	Total creatine.	Preformed creatinine	Total creatinine.
1	Cardiac	1.0	9.8	0.95	5.7
2	Arthritis	1.0	10.0	1.00	6.6
3	"	0.98	9.2	1.00	6.6
4	Cardiorenal disease	1.30	12.0	1.00	8.0
5	Cardiac	1.10	7.5	1.00	5.7
6	Gastric ulcer	1.0	9.0	0.95	6.5
7	" "	1.2	8.8	1.00	6.5
9	Hernia	1.2	10.0	1.00	6.5
10	"	1.3	12.0	1.00	6.3
11	Pneumonia	1.1	9.0	1.00	6.3
12	Fractured femur	1.1	8.3	1.10	6.2
13	Syphilis	1.1	8.2	1.00	6.3
14	Carcinoma	1.3	7.1	1.00	6.0
15	Arthritis	0.95	7.7	0.9	4.0
16	Pleurisy	1.0	9.0	1.0	5.5
17	Cardiorenal disease	1.2	9.2	1.0	6.0
18	Chronic constipation	1.1	8.4	0.9	5.0
19	Arthritis	1.0	7.9	1.0	5.6
20	Syphilis	0.98	8.8	1.0	5.0
21	Cardiorenal disease	1.10	11.0	1.0	7.0
22	Arthritis	1.00	10.0	0.9	4.6
23	Cystitis	1.20	9.2	1.1	6.0
24	"	1.20	9.9	1.2	5.8

lined and obtained quantitative recovery; in the light of our present knowledge it must therefore be concluded that the method of protein precipitation used has eliminated, to a certain extent at least, the reducing bodies other than creatinine to the presence of which has been attributed the high results obtained by the picric acid precipitation.

FAT-SOLUBLE VITAMINE. I.*

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(From the Laboratory of Agricultural Chemistry, University of Wisconsin Madison.)

(Received for publication, July 8, 1918.)

In 1916, it was reported from this laboratory that the fat-soluble vitamine could be separated from melted butter fat by shaking it with successive portions of water.[1] This statement was justified only in part. We have since found that while the vitamine was removed from the washed butter fat the washings did not contain it. Somewhere in the course of the manipulations to which the butter fat had been subjected factors had been introduced which were responsible for a vitamine destruction. These observations were surprising as the idea is quite generally prevalent that the fat-soluble vitamine is stable.[2] As far as the authors are aware only one case has been reported in which a lability of the vitamine was observed. This observation was made by Osborne and Mendel[3] working with butter "oil." In butter fat under their conditions they found it entirely stable. In our next experiment, we tried shaking the melted butter fat without change of water. After 12 hours of this treatment, the water was separated from the fat, brought down *in vacuo*, and such residue as it left was united with the treated fat. This also gave an entirely inactive preparation. Aerating butter fat at 100° for 12 hours led to the same result; the vitamine had been completely destroyed. Later experiments showed that in the

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[1] McCollum, E. V., Simmonds, N., and Steenbock, H., *J. Biol. Chem.*, 1917, xxix, p. xxvi.

[2] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913–14, xvi, 423; 1915, xx, 379. McCollum, E. V., and Davis, M., *Proc. Soc. Exp. Biol. and Med.*, 1913–14, xi, 101.

[3] Osborne and Mendel, *J. Biol. Chem.*, 1916, xxiv, 37.

shaking process the vitamine was not always completely destroyed for reasons which will be evident later.

At one time we were inclined to consider the process leading to the destruction of the vitamine as an oxidation phenomenon, but the evidence obtained from feeding butter fats shaken with carbonated water did not strengthen this point of view. The manipulations were not controlled carefully enough, however, to decide this point definitely.

We now realize as we did not before that heat alone in the absence of water or in the absence of conditions designed to bring about intimate contact with air was responsible for the vitamine destruction observed in the early experiments. In all of these the butter fat had been heated and thus the destruction of its vitamine brought about. When butter fat is heated for 4 hours at 100°, so much of its vitamine may be destroyed that with our basal rations, using rats as the experimental animal, no demonstrable amounts of the vitamine remained.

The failure of other investigators to note this destructive action was undoubtedly due to the high initial content of fat-soluble vitamine in the material studied. The destructive process is evidently a reaction of low velocity. With large amounts of vitamine present and with heat treatment for a limited period of time, sufficient amounts of the vitamine remained to satisfy all the requirements for normal growth in the experimental animals. The most favorable conditions for such observations obtain when the amount of vitamine is barely sufficient for normal or even slightly subnormal growth. These conditions obtained in Ration 306 as shown in Chart 6. That the destructive action of heat was considerable is furthermore shown by comparison of the growth of these animals with those of Ration 307, Chart 7, where the unheated butter fat was fed at a level of only 5 per cent of the ration. The heated butter fat fed at a 12 per cent level was far less efficient than the unheated fat fed at a level of 5 per cent.[4]

[4] This comparison is made in full realization of the fact that the energy content of Ration 306 with its larger fat content is greater than that of Ration 307. Assuming that the relative consumptions of the ration would be proportional to the energy requirements of the animals, the vitamine intake on the ration containing 5 per cent of butter fat would be greater than that indicated when expressed on the percentage of weight basis. It would be much more logical to express it on the calorie basis. This is

In the course of many experiments designed to demonstrate the vitamine content of butters we have been impressed with variations in the amount of vitamine present. These variations are not the result of heat treatment as in some cases the exposure to heat in the preparation of the fat was reduced to the minimum necessary to secure a clear sample. We surmised that possibly the rations on which the butter fat had been produced might be responsible. On these general premises lard could be said to owe its deficiencies in vitamine to the poverty of the grains in this dietary essential. Similarly beef fat would owe its correspondingly greater vitamine content to the greater vitamine content of the roughages such as clover and alfalfa so extensively fed for beef and milk production. As yet experiments in this direction have not progressed sufficiently to warrant generalization. One fact, however, is clear. Vitamine content of the ration on which butter fat is produced is not the only factor to be considered. One sample of butter fat obtained from a cow fed exclusively on alfalfa hay was found to contain no demonstrable amounts of vitamine. This butter had been kept in an unsalted condition in a poorly iced refrigerator for about 3 weeks. In this time some molds had developed on its surface which necessitated discarding part of the material.

Much emphasis has been placed on the superior value of butter fat as compared with other fats used in the human diet, due to the larger content of fat-soluble vitamine in the former. The experiments briefly discussed in this paper indicate some of the conditions which may modify and even eliminate all reasons for this distinction. Lard, vegetable fats, and beef fats on the average, as has been shown repeatedly, do not compare favorably with butter fat. The liquid portion of beef fat as first found by Osborne and Mendel[5] and later by Halliburton and Drummond[6]

in harmony with Osborne and Mendel's statements (*J. Biol. Chem.*, 1918, xxxiv, 522) that in order that comparisons of effects of different rations may be warranted the amount of the rations consumed must be controlled. Palatability and bulkiness as well as available caloric content are factors which are important in determining the total amount of a ration consumed. Allowances made for variations in the amounts of the rations consumed in the experiments reported in this paper would not affect the validity of any of the conclusions drawn.

[5] Osborne and Mendel, *J. Biol. Chem.*, 1915, xx, 379.

[6] Halliburton, W. D., and Drummond, J. C., *J. Physiol.*, 1917, li, 235.

and also the fats of certain animal organs[7] are richer in the fat-soluble vitamine and in some instances, as shown in Charts 14 and 15, may compare very favorably with butter fat. In general, however, oleomargarines on account of the large amounts of the vitamine-poor fats used in their manufacture and in spite of the fact that they are churned with milk and contain some butter fat are not to be considered in the same class as good butter in providing the organism with the fat-soluble vitamine. Questions of influence of feed, storage conditions, and temperatures used in the renovation of inferior products and even the method of use of the product in the home must be taken into consideration when depending upon butter fat as a sole source of fat-soluble vitamine in the dietary. With an abundance of a considerable amount of the vegetative parts of plants in the diet the superior value of butter fat over other fats in the human diet is questionable. In the rat we have found as small an amount as 5 per cent of alfalfa in the ration providing sufficient fat-soluble vitamine for normal growth, reproduction, and the rearing of some young. In the absence of such materials in the diet the superior value of butter fat and in fact its necessity therein for normal nutrition is not to be denied. Then it becomes an important matter to know how this superior quality of butter fat can be obtained and preserved.

Charts of growth curves of rats used in these preliminary experiments follow.

[7] Osborne and Mendel, *J. Biol. Chem.*, 1918, xxxiv, 17.

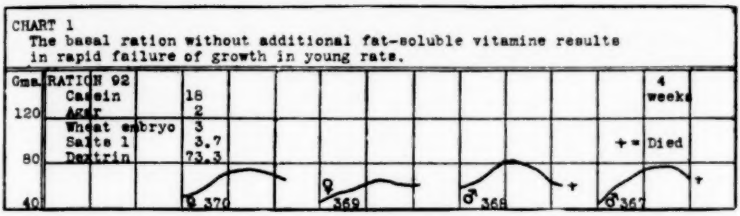

CHART 1. Ration 92. In this lot and in all the succeeding lots young actively growing rats of from 40 to 70 gm. weight were selected. In the rations fed the casein was a commercial article purified by washing repeatedly with dilute acetic acid. The wheat embryo was ether extracted. The salt mixture was of the same composition as that referred to in a previous publication (*J. Biol. Chem.*, 1918, xxxv, 61). As therein stated the salt mixture is more complex than necessary. For example, we have had excellent growth, reproduction, and rearing of young on rations free from sulfates. As in these experiments we were not concerned with the inorganic relations of the diet beyond covering the requirements of the animals, its use was continued. The dextrin was prepared by heating cornstarch with a dilute citric acid solution for 3 hours at 119°.

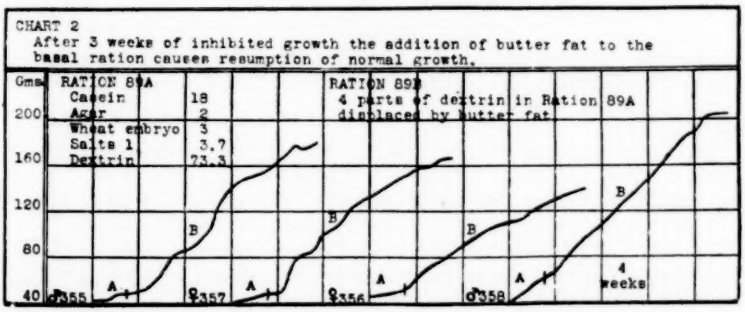

CHART 2.

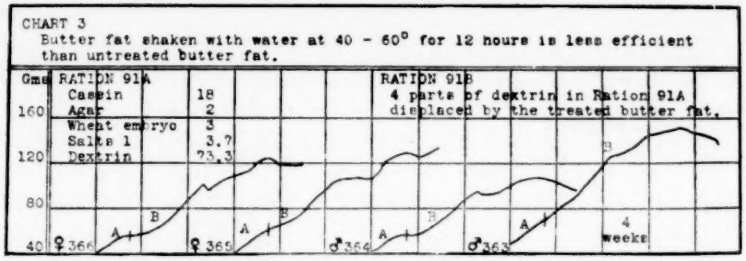

Chart 3. Ration 91B. The butter fat fed in this lot was shaken violently in a large glass-stoppered bottle with about a liter of warm distilled water. Whenever the temperature fell so that butter fat tended to solidify, the bottle with contents was emersed in a pail of hot water until the temperature had risen to about 60°. Here the temperature was not carefully controlled as its importance in the destruction of the fat-soluble vitamine had not been recognized. Comparison with Chart 2 clearly indicates a decreased efficiency of the treated butter fat in supplying the required amounts of the fat-soluble vitamine.

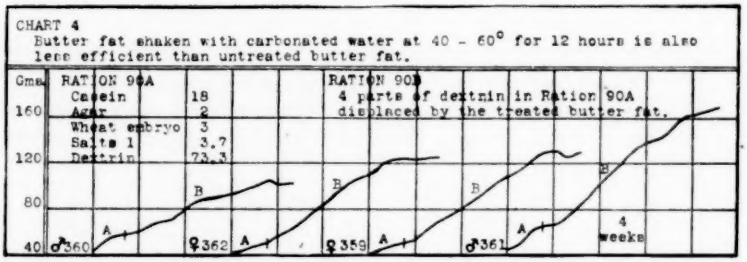

Chart 4. Ration 90B. With the air in the bottle displaced by carbon dioxide the shaking process at 40–60° as carried out in the previous experiment (Chart 3) was apparently just as destructive. We had been inclined to consider the destruction of the fat-soluble vitamine as an oxidative reaction, but this did not justify that opinion.

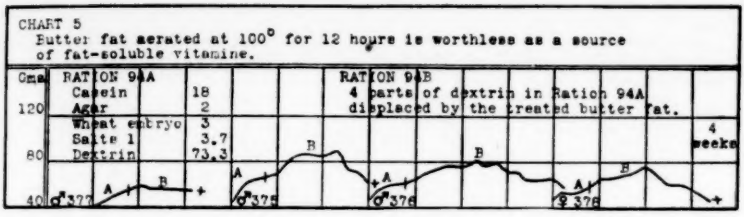

CHART 5. Ration 94B. The butter fat used in this ration was aerated by drawing through it a current of air washed with dilute acid and alkali and then filtered through cotton.

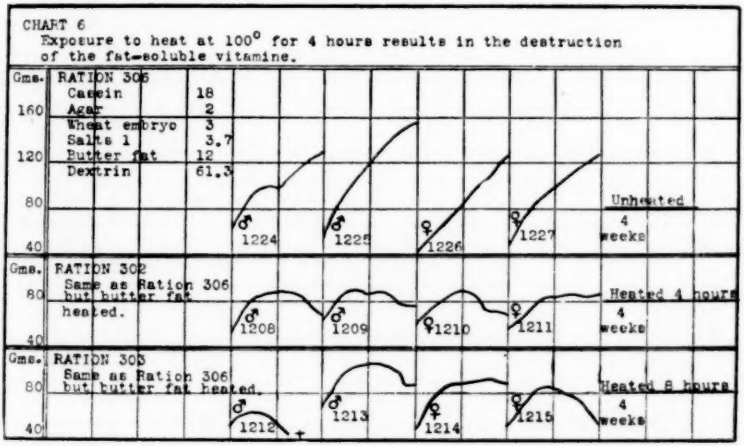

CHART 6. Rations 306, 302, and 303. In this series of experiments the rats were not fasted for fat-soluble vitamine before being placed on the butter fat rations. We previously were of the opinion that the preliminary period of fasting might make the rats more responsive to the change in ration, but this was not found to be the case. The heated butter fats here employed were heated in pint mason jars at 100° in a Freas oven. With no excessive amount of the fat-soluble vitamine present in Ration 306 which served as a control the destructive action of heat was very apparent. How much the period of exposure might have been abbreviated or the temperature lowered and yet give the same results we have not yet determined. The experimental animals on Rations 302 and 303 at the end of the 8 weeks period were in a miserable condition, nearly all having been blinded by a purulent conjunctivitis.

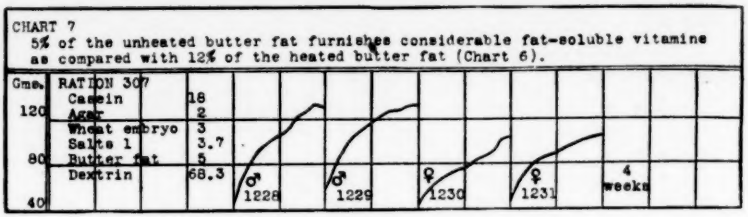

Chart 7.

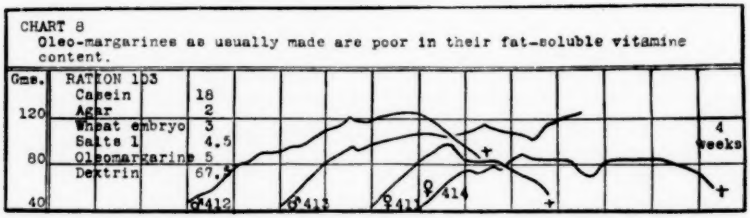

Chart 8.

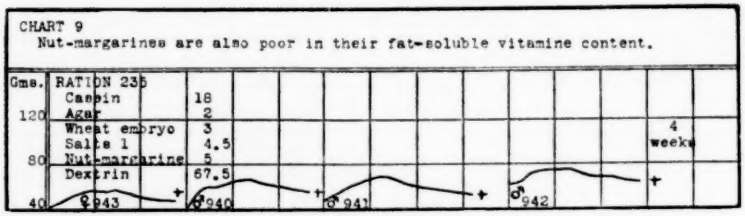

Chart 9.

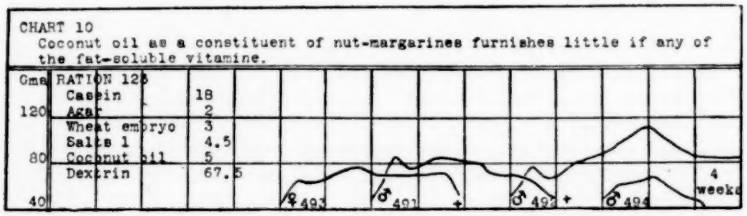

Chart 10.

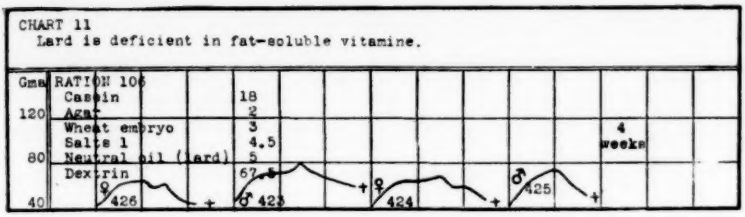

Chart 11.

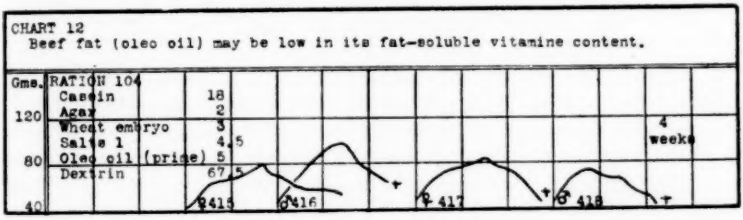

Chart 12.

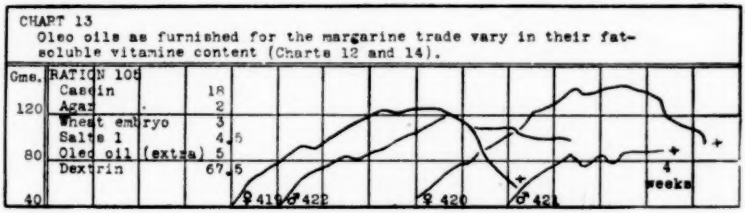

Chart 13.

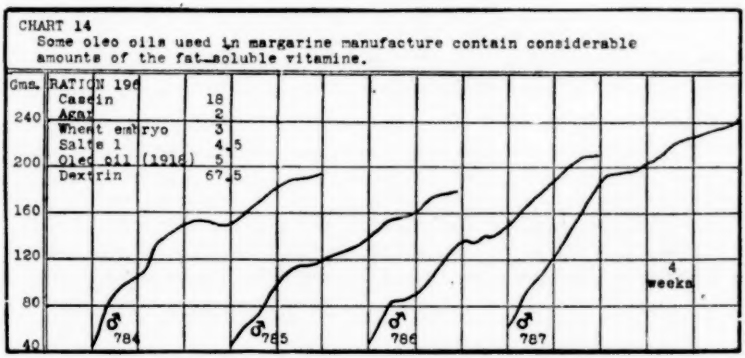

Chart 14.

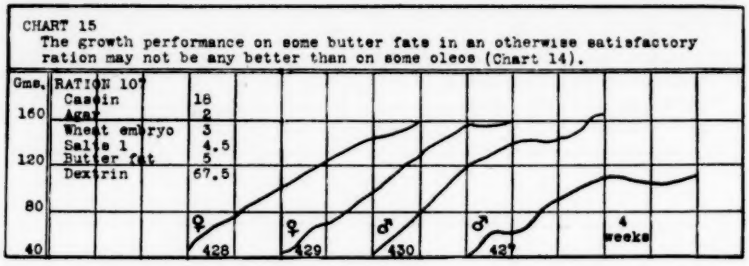

CHART 15. Ration 107. We have had numerous instances where 5 per cent of butter fat used as the sole source of fat-soluble vitamine in an otherwise fat-poor ration has not furnished sufficient amounts of this vitamine for normal growth. As used in our experimental work butter obtained from the University creamery is melted in a steam oven at about 95° and kept at this temperature for 15 to 45 minutes, when the butter fat is decanted on a folded filter and filtered rapidly. Filtration is facilitated by keeping the butter fat warm at about 50° by means of a water-jacketed funnel.

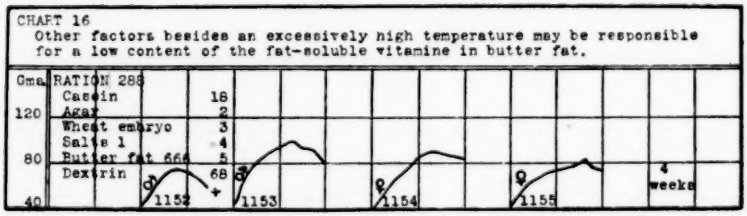

CHART 16. Ration 288. The butter used in this ration as the source of butter fat had been kept unsalted under poor storage conditions. It was obtained from a cow fed exclusively on alfalfa, and therefore a high fat-soluble vitamine content was surmised. As demonstrated by the poor growth performance of the rats, such did not prove to be the case. Other conditions aside from an excessively high temperature may be responsible for a low content of the fat-soluble vitamine in butter fat.

THE REFRACTIVE INDEX OF THE BLOOD SERUM OF THE ALBINO RAT AT DIFFERENT AGES.

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In connection with the work on the metabolic activity of the central nervous system it was thought desirable to determine at the same time the chemical composition of the blood. With hope that the main changes under experimental conditions might be quickly revealed, preliminary examination of the blood serum was made by means of the refractometer. A considerable difference was observed in the value of the refractive index given by the younger and older albino rats. Further study not only confirmed my preliminary observations, but it was found that the changes in the refractive index of blood serum due to age are quite large, and at the same time the course of change is remarkably regular. The data on this refractivity of the serum show several points of biological interest and suggest the need of further extensive studies along this line.

Materials and Technique.

The Pulfrich refractometer[1] was used for the present investigation and the tests were made with sodium light. The temperature was so regulated that the serum in the receiving chamber is within 20–23°C. If the temperature was either higher or lower than this, a correction was made for the reading of the scales. After careful tests it was found that the variations of the refractive index of the serum, if examined within these limits of the temperature, are practically insignificant and therefore no correction is necessary, at least for the present purpose.

[1] A concise theoretical account with many helpful suggestions on the use of the Pulfrich refractometer is given by Findlay (1914).

Albino rats were mainly used, but in a few instances hybrids of the albino and also Norway rats were employed. The rats were kept on the ordinary laboratory diet and the blood was taken before feeding; that is, the rats were fed twenty-four hours previous to the withdrawal of the blood. Only in the cases of the younger rats, which were still nursed by the mother, were the animals taken at any convenient time. In several instances these suckling rats were isolated from the mother for variable periods (10 to 16 hours) to bring them into a condition somewhat similar to that of the grown rats. As will be seen from the table, there is no material difference in the amount of refraction whether the young rats were isolated from the mother for several hours or taken while their stomachs were still filled with milk. A slight amount of fat globules or a slight hemolysis does not apparently modify the refractive index of the serum to any significant extent.

The blood was obtained in the following manner. The rats were lightly anesthetized with ether and the thoracic cavity was quickly opened and the blood at once taken from the heart. In the case of the small rats, however, the blood was taken by severing the carotid artery. In order to avoid any admixture of the body fluids no pressure was applied, but only spontaneously dropping blood was received. The bottle was then closed to avoid evaporation and left at room temperature until the serum had separated. Then, by means of a pipette, the serum was transferred to a small test-tube and the blood corpuscles were completely separated by means of the centrifuge. The serum thus obtained is now ready for a determination of the refractive index. The amount of serum necessary for a determination is about 0.5 cc. or even less. The excess of the serum obtained was dried at a temperature of 95°C. for 1 week and the water content was determined. In some instances a small amount of toluene was used to prevent any evaporation during the determination of the refractive index.

Refractive Index of Blood Serum at Different Ages.

Altogether 35 groups consisting of 213 normal albino rats of various ages were used for the present investigation. This large number was required owing to the small yield of blood serum in

the case of the younger rats. As only a drop or two was to be obtained from new born rats and even in rats 1 week old, in which the body weighs nearly 10 gm. or more, the desired amount of the serum is obtained by using five to seven rats at once. This disadvantage represented by the necessity of taking large numbers of rats is however fully compensated by the fact that we can thus eliminate individual variations to a considerable extent. The main results obtained from this investigation are shown in Table I.

In Table I a value of the refractive index of the serum N_D is also given, from which the value of the refractive index of distilled water at 17.5°C. ($N_D = 1.33320$) was subtracted and the resulting value multiplied by 1,000. This value thus obtained is usually written $\Delta N_D \times 10^3$ to which I have given the term "micro-refractive index." This micro-refractive index is convenient for comparison, and in fact some authors give this index alone. I have subtracted the constant value of 1.33320 instead of subtracting the value of water at the time of examination,—for if a constant value was always used, the restoration to the original value of the refractive index can be made at once. If, on the other hand, the water value at the time of the experiment was subtracted, such restoration is impossible unless the refractive index of the water was also given in each instance. In several papers this micro-refractive index is given without stating the data on the water, the refractive index of water at the time of examination having been subtracted. As long as the temperature at the time of the determination of the refractive index of the serum was within the limit of 20–23°C. or even lower, the standard value may be used without correction for the temperature, since the error produced from this source is negligibly small for work of this nature. For convenience in studying the changes in the refractive index of the serum at different ages, the data of Table I are plotted in Chart 1.

As will be seen from Chart 1, the value for the refractive index of the serum rises very rapidly up to about 90 days and then becomes slow, though rising continuously. We note also some irregularities in the distribution of the refractive index at the age of about 20 days and again at about 85 days of age. These irregularities in the distribution, as well as sudden change in a

TABLE I.

Refractive Index of Blood Serum Together with Percentage of Water in the Serum of the Albino Rat at Different Ages. 35 Groups.

Body weight.	Length.	Sex.	Age.	No.	Blood serum.				Remarks.
					Water.	Solids.	N_D	ΔN_D	
gm.	*mm.*		*days*		*per cent*	*per cent*			
5.1	—	Mixed.	New born.	8	95.90	4.10	1.34136	8.16	Clear.
7.0	—	"	3	15	95.88	4.13	1.34132	8.12	Very cloudy.
8.4	—	"	5	21	95.76	4.24	1.34261	9.41	Removed from mother, clear.
10.4	—	"	7	15	95.77	4.23	1.34266	9.46	Cloudy.
13.8	—	"	10	20	95.30	4.70	1.34340	10.20	Removed from mother, clear.
16.6	—	"	12	3	94.71	5.29	1.34454	11.34	Cloudy.
18.9	—	"	15	17	94.19	5.81	1.34509	11.89	"
23.9	—	"	17	9	94.01	5.99	1.34575	12.55	"
26.9	—	"	18	6	93.82	6.18	1.34632	13.12	"
28.3	93	"	19	10	93.89	6.11	1.34579	12.59	"
23.7	—	♀	20	3	93.70	6.30	1.34598	12.78	"
29.8	98	Mixed.	21	4	—	—	1.34577	12.57	"
37.0	105	"	23	8	94.03	5.97	1.34549	12.29	"
35.9	108	"	26	9	93.92	6.08	1.34596	12.76	Clear.
47.2	115	♀	30	2	93.92	6.08	1.34568	12.48	"
52.7	129	Mixed.	37	4	94.02	5.98	1.34581	12.61	"
67.9	145	"	41	2	93.95	6.05	1.34619	12.99	"
82.1	149	♂	44	1	—	—	1.34649	13.29	"
78.9	148	Mixed.	50	3	93.27	6.73	1.34732	14.12	"
—	154	"	52	2	—	—	1.34727	14.07	"
78.9	151	♀	60	1	—	—	1.34794	14.74	"
94.9	158	Mixed.	66	6	92.74	7.26	1.34831	15.11	"
109.4	164	♂	69	4	92.71	7.29	1.34836	15.16	"
—	—	Mixed.	72	3	—	—	1.34868	15.48	"
—	—	"	74	2	—	—	1.34863	15.43	"
134.7	176	"	80	6	92.27	7.73	1.34873	15.53	"
138.0	181	"	85	5	92.44	7.56	1.34851	15.31	"
134.6	179	"	92	4	92.07	7.93	1.34959	16.39	"
—	192	"	100	3	—	—	1.34958	16.38	"
176.5	192	♂	120	6	92.00	8.00	1.34965	16.45	"
196.8	201	♂	161	2	91.75	8.25	1.35006	16.86	"
—	—	Mixed.	174	3	—	—	1.35037	17.17	"
—	—	♀	365	2	—	—	1.35123	18.03	"
166.4	205	♀	450	2	91.22	8.78	1.35115	17.95	"
250.0	222	Mixed.	597	2	—	—	1.35175	18.55	"

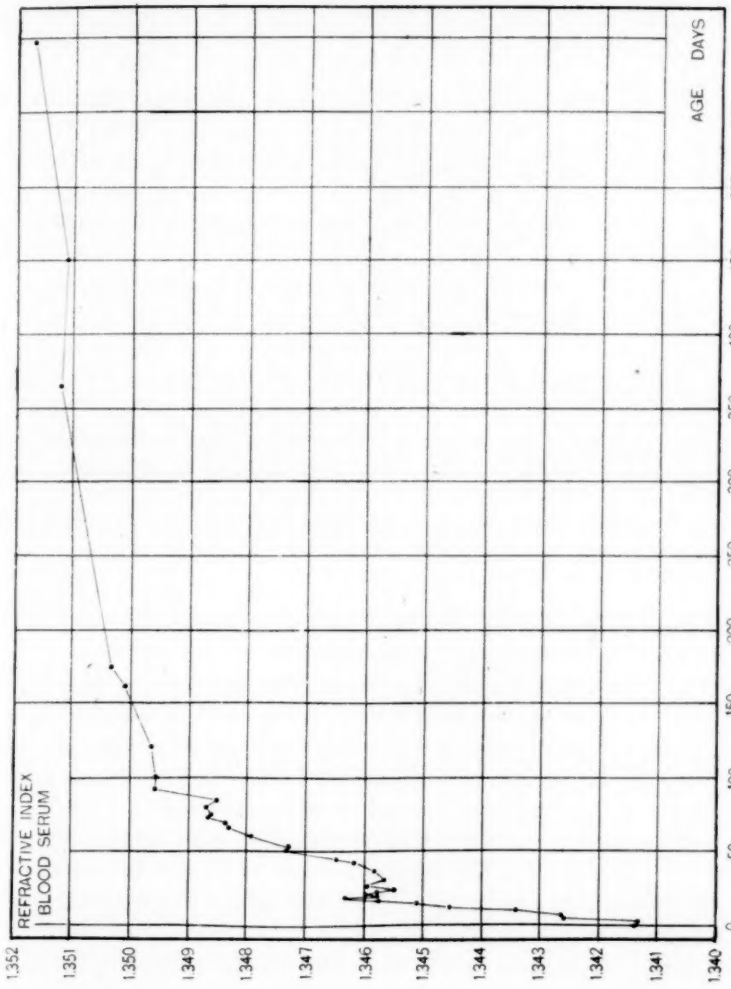

CHART 1. Showing the refractive index of blood serum at different ages.

general course of the graph, coincide very well with the following three important periods of the life cycle of albino rats; (1) the suckling period or about the first 23 days, (2) from the end of the suckling period to puberty or about 85 days old, and (3) from puberty to the end of the record. A discussion of these periods follows.

1. The Suckling Period.—The refractive index at birth is 1.34136, slightly higher than at 3 days, which gives a value of 1.34132. This higher refractive index is interesting since the new born rat previous to suckling possesses blood, the composition of which is largely regulated by the placenta and which differs also from that of the maternal blood.

Abderhalden and Weil (1912) found in the serum of the ox and of the fetal calf that the former polarizes the light more strongly than the latter. In the human subject these two authors also found that the blood serum taken from the umbilical cord polarizes the light noticeably less than the serum of the mother. The nitrogen content of the serum of the fetus is far less than that of the mother. Abderhalden and Weil give the following data.

1 cc. ox serum contained	0.0144 gm.	N.	Polarization	−0.83°
1 " sheep " "	0.0134 "	N.	"	−0.74°
1 " fetal calf serum contained	0.0059 "	N.	"	−0.27°

All these facts indicate that the blood serum at or before birth differs from the blood serum of later life.

Reiss (1909) determined the refractive index of the human serum at different ages, together with the protein content of the serum. Reiss found also that the refractive index of the new-born is much higher before suckling than after; *i.e.*, the index for the serum of the new-born before suckling is, 1.34746, while at 7 and 11 days the indices are 1.34668 and 1.34648 respectively. Thus my own observation on the rat agrees with those of the human infant in regard to the higher value of the refractive index of the serum before suckling.

Following this initial fall in the value of the refractive index there is a rapid rise after suckling until the rat is 18 days old (N_D = 1.34632), and then falls again until the 23rd day (N_D = 1.34549). As will be seen from the chart, the index values are very regular up to about 18 days, then they become irregular

till about 30 days of age, after which they are again regular. From numerous observations the suckling period of the rat is considered to be approximately 21 days, although there must be some variation in the age at which the rat begins to take some solid food and becomes independent of the milk diet. It was found however that the serum is usually more or less cloudy, owing to the admixture of milk fat, up to 18 days and this cloudiness diminishes daily until about the age of 23 days, and after this the serum is usually perfectly clear if the blood is not examined immediately after feeding. This fact indicates that the young rats are not taking any large quantity of milk after 23 days of age, but probably solid food mainly, even when they are still with the mother. We may infer also from the relative degree of cloudiness of the serum that the young rats begin to take some solid food at about 17 or 18 days. The period at which the young rats begin to take solid food, or to take such food exclusively, must largely depend on the relative degree of maturity of the individuals. It was found that the refractive index of the serum differs quite considerably whether the mother had a large number in the litter or a few (see page 544). The irregularity shown in the period between 18 and 30 days may therefore be due to different degrees of growth among young rats, and also to the adjustment of the rat to change of diet.

The mother has sometimes been separated from the suckling rats for 10 to 16 hours in order to obtain a completely clear serum. A comparison of such clear serum with that more or less clouded, shows no appreciable difference in the value of the refractive index. This seems to indicate that the presence of fat globules does not significantly alter the refractive index.

I am unable to explain how the presence of a small amount of solid food in the digestive tract at the end of the suckling period, or soon after suckling has ceased, alters the refractive index of serum, but this alteration seems to involve not only the relative amount of the serum proteins, but also the percentage of water in the serum.

Table III shows that the water content of the entire blood at birth is 83.0 per cent, but it rises to 86.8 per cent at 15 days, after which it falls off, at first rapidly and then slowly (79.3 per cent at 1 year old). In this respect the changes in the entire

blood agree with the variations shown by the specific gravity of the entire blood and indicate that the presence of the blood corpuscles is in part responsible for this change in the percentage of water.

We also notice from Chart 2 that the percentage of water falls rapidly up to 21 days with the exception of the first 7 days, during which period the diminution of water is somewhat slower. This slower rate in the diminution of water during the first 7 days coincides with the adjustment period of the rat from the placental stage to the first experiences in the postnatal life. The refractive index of the serum reaches the first maximum height at the end of about 18 days, while the percentage of water reaches a minimum at 21 days, and, furthermore, the percentage of water falls without interruption up to this age (21 days). This slight discrepancy in the percentage of water on the one hand and the refractive index on the other may be due to the presence of fat globules in the serum which would influence the percentage of water while having little influence on the values of the refractive index.

The actual determination of the water content of the serum at this critical period shows changes which run parallel to those in its refractivity (for a more detailed discussion see page 538). In general we may conclude that with the change from mother's milk to a solid food some alteration takes place in the composition of the serum and complete adjustment to this change is reached only several days after the rats become entirely independent of the mother. It is worthy of note that during the rather considerable period of alteration in the refractive index of the serum the growth of the body as a whole does not show any corresponding alteration, but growth in weight, as well as in length, continues steadily (Donaldson, 1915).

2. From End of Suckling Period to Puberty.—Soon after the adjustment to the changes in diet, the refractive index of the serum rises rapidly, though its rate is not as great as during the first 18 days of life, and it continues to rise until about the age of 85 days. Between 70 and 85 days the refactive index again becomes irregular. This second irregularity can hardly be a mere statistical result since the number of cases examined was large, and furthermore an extreme regularity in the distribution of the index is shown immediately before and after this period. These

results suggest that this is another critical period at which some important changes in the composition of the serum are taking place. We have abundant data to show that this is about the period of beginning sexual activity and the rats may begin to breed at this period or a little later. For this reason I am inclined to believe that this irregularity in the refractive index of the serum is somehow related to the beginnings of sexual life. As will be seen from Chart 1 this condition at 85 days is transitional to the last phase in the span of life.

3. From Puberty through the Remainder of Life.—As is shown in Chart 1, after the rat has reached about 90 days, the subsequent alteration in the value of the refractive index is small, though it continues to rise throughout the rest of life so far as plotted. It must be stated however that after 200 days the refractive indices given by individual rats become highly irregular owing to the onset of lung infection, and the irregularity increases with advancing age owing to the presence of various metabolic or organic troubles. The data in Chart 1 were taken from the rats which were entirely free from pathological alteration so far as could be determined. It is very difficult to find perfectly sound rats at the age of 200 days or more, but, when such rats are examined, the refractive index of their serum is astonishingly uniform.

Refractive Index of Human Serum Compared with That of the Albino Rat.

So far I have presented the observations on the changes in the refractive index of blood serum made exclusively on the albino rat. There are however some isolated records on the refractive index of human serum, but unfortunately most of these data were taken from hospital subjects and cannot be used except for special purposes. To the best of my knowledge there are only two papers which deal with the refractive index obtained from the normal human serum. One contains the observations made by Reiss (1909), who determined the refractive index of the serum taken mostly from younger children, and the second paper is that of Gettler and Baker (1916) who made an elaborate analysis, chemical and physical, of the normal human serum of thirty adult

individuals. The combination of these two studies covers the most important part of the life span of man (from birth to 55 years) and affords an opportunity for comparison of my own data on the rat with those for man. Table II is based on the observations of Reiss and of Gettler and Baker.

TABLE II.

Refractive Index of Man at Various Ages. Data from Reiss (1909) and Gettler and Baker (1916).[2]

Age.	N_D	Remarks.	Body weight.	Sex	Age.	ΔN_D	N_D	Remarks.
			lbs.		*yrs.*			
1½ days.	1.34746	Before suckling.			9½		1.34978	
7 "	1.34668	"			11		1.34964	
11 "	1.34648	Breast feeding.			12		1.35046	
13 "	1.34557	"			13		1.34992	
19 "	1.34693	"			14		1.35030	
42 "	1.34580	"			18		1.35071	
2 mos.	1.34654	"	108	♂	17	16.4	1.34960	1
3 "	1.34647	"	138	♂	19	18.9	1.35210	1
4 "	1.34740	"	144	♂	20	17.8	1.35100	1
4½ "	1.34627	"	169	♂	21	17.05	1.35025	4
5 "	1.34722	"	177	♂	22	17.3	1.35050	2
5½ "	1.34762	"	162	♂	23	17.6	1.35080	2
8 "	1.34976		131	♂	25	17.5	1.35070	1
10 "	1.34733		139	♀	26	15.8	1.34900	1
12 "	1.34920		184	♂	28	17.2	1.35040	2
14 "	1.34895		136	♂	38	18.1	1.35130	1
22 "	1.35110		220	♂	39	18.4	1.35160	1
2 yrs.	1.34966		118	♂	45	16.6	1.34980	1
3 "	1.35060		145	♀	45	16.3	1.34950	1
4 "	1.34907		124	♀	48	18.8	1.35200	1
5 "	1.34920		145	♀	50	16.2	1.34940	1
6 "	1.35001		180	♂	55	17.0	1.35020	1
7 "	1.34909							
8 "	1.34987							
9 "	1.34986							

[2] Several cases in which the serum gives a positive Wassermann reaction as well as some doubtful by this test were excluded. Gettler and Baker give the micro-refractive index $\Delta N_D \times 10^3$ but I have changed their values to the value of N_D by adding 1.33320. Any changes arising from this correction should be very slight and thus should not affect the conclusions drawn from them.

As we should anticipate the data on man are highly variable when compared with those for the rat, owing to numerous but unavoidable technical difficulties connected with the study of human material. However, we obtain three important facts concerning the refractive index of human serum which are clearly marked. (1) According to the table by Reiss, the index is higher at birth (before suckling) than after suckling; that is, the index of the serum falls off rapidly for the first 2 weeks, and afterwards it rises again. The index at $1\frac{1}{2}$ days after birth is 1.34746, and 13 days after, it sinks to 1.34557. (2) After this initial fall of the index it rapidly rises until the 6th or 7th month. From this period on it gradually and slowly rises up to 55 years (upper limit of observations on the male subject). (3) The adult male tends to give a higher refractive index than the female (see Table II). No similar observations on sex are available before the age of 25 years.

These facts agree essentially with those found for the serum of the albino rat, in which the refractive index of the serum at birth is slightly higher than some days after suckling. Following this initial fall, the index rises rapidly to the end of the suckling period. After adjustment to change of food, the index continues to rise rapidly to the completion of sexual maturity. From the completion of sexual maturity the refractive index continues to rise but slowly throughout the remainder of the life span, so far as examined.

It is interesting to note that the value of the refractive index given by human adults is approximately the same as that given by adult albino rats, as the following relation shows. After 90 days the increase in the refractive index becomes very slow.

Rat. *days*	N_D	N_D	Man. *yrs.*
90	1.34999	1.35071	18
550	1.35175	1.35160	39

The very high refractive index of man at birth, compared with that for the rat at birth and before puberty, is difficult to understand at this moment.

Recently Wells (1913) studied the relative proportions of the serum proteins in rabbits at different ages, together with the influence of diet on these proportions. Wells found that the serum

protein in the rabbits increases with age from 21 days to 149 days, but that fully adult animals have a slightly lower content of proteins in their serum than animals which are between 100 and 150 days old. This decline in the serum protein after the animal has reached the full adult stage is rather unexpected to the present writer, since the work of Reiss (1909) and of Gettler and Baker (1916) combined shows an unmistakable increase of the proteins with increasing age. My own data on the rat also indicate indirectly a continuous increase of the total proteins with increasing age, as can be inferred from the continuous increase of the refractive index. Since the amount of serum proteins seems to vary with the state of the animal's health, one may perhaps question whether or not the fully adult rabbits used by Wells were entirely normal.

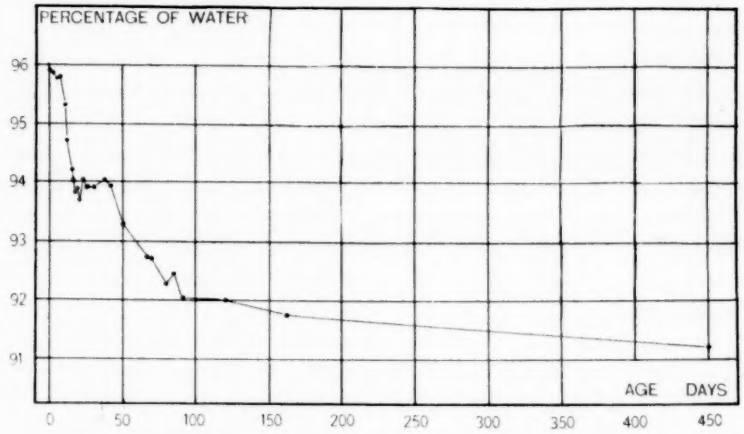

CHART 2. Showing the percentage of water in the serum of the albino rat at different ages.

Water Content of Serum.

The percentage of water in the serum varies according to the age of the rats in nearly the same manner as the refractive index, but inversely. Therefore we may similarly divide the entire graph for the water content into three phases. The total number of rats used for the water determination of the serum was 190, and the values given by respective ages are shown in Table I. A graph of the percentage of water in the serum based on the data given in Table I is shown in Chart 2.

We notice that the content of water in the serum diminishes with the increasing age of the rat. In this respect the serum behaves like the other tissues or organs. This datum is however to be carefully distinguished from the water content of the entire blood. I have made a short series of observations on the percentage of water in the entire blood of the albino rat and found the relations given in Table III.

2. From End of Suckling Period to Puberty.—This rapid fall in the water content is followed by a nearly stationary period for almost the next 20 days, which coincides with the period of readjustment to the change in the diet, and in turn is followed by a rapid fall in the water content up to about 85 days, which marks the completion of sexual maturity.

TABLE III.

Percentage of Water in Entire Blood of the Albino Rat at Different Ages.

Body weight.	Body length.	Age.	Sex.	Percentage of water.	No. of rats.
gm.	*mm.*	*days*			
4.2		New born.	Mixed.	83.0	8
15.5	77	15	♂	86.8	4
66.4	139	45	♂	81.9	2
173.8	192	108	♂	79.9	2
306.9	235	365	♂	79.3	2

3. From Puberty through Remainder of Life.—After 85 days of age we notice only a very slight fall in the percentage of water during the rest of the life span, so far as examined. Thus there appears a very close connection in the changes in the percentage of water and the changes in the refractive index of the blood serum.

Data on the water content of the serum may be contained in the medical literature, but, so far as I am aware, no systematic investigation has been made on any one species of animal in which the ages have also been recorded. Hock and Schlesinger (1892) give a short report on the specific gravity of human serum, from which we take the following records.

	Specific gravity.
Suckling infant	1.0265
Child, 2 yrs.	1.0300
" 7 "	1.0315

Reiss (1909) also states that the blood serum of the nursing baby is poorer in solids and richer in water than in the adult, but gives no data.

These observations on the human serum are in harmony with changes in the percentage of water in the albino rat, and we may conclude therefore that the water content of mammalian serum diminishes with increasing age.

Percentage of Solids in the Serum in Relation to Refractive Index.

Inasmuch as the variations in the percentage of water of the serum are linked with the variations in the refractive index of the

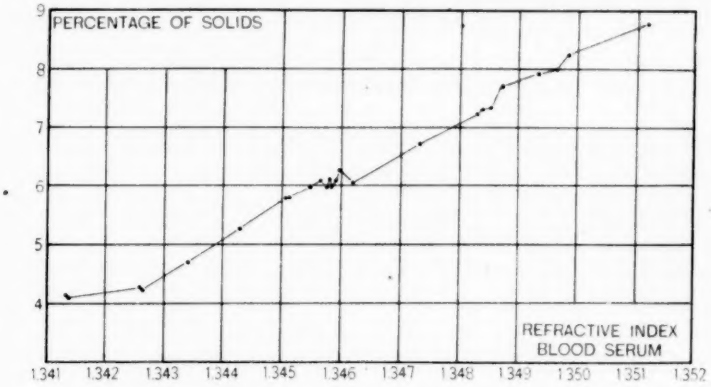

CHART 3. Showing the variations in the percentage of solids accompanying the increasing values of the refractive index of serum.

rats at corresponding ages, we may anticipate that the relation between the refractive index and the percentage of solids may also be regular and constant. To show this relation clearly the data of Table I are plotted in Chart 3.

From Chart 3 it is seen that the relation between the percentage of solids and of the refractive index is regular for the most part, and is almost linear. We do find however some discordant relations during the first 7 days of life; that is, the increase in the percentage of solids during this early period is disproportionately small. This may mean that at this early period though the proteins of the serum are increasing rapidly, as can

be seen from the rapid increase in the refractive index (Chart 1), yet some substances, such as fat globules, lactose, etc., are present in but slight amount, thus producing a smaller value for the solids. For the explanation of this phenomenon we must however await further study. After this period the relation between the percentage of solids and the refractive index is practically linear. The slight irregularities shown in the percentage of solids at the region where $N_D = 1.34600$ are probably due to the presence of fat globules in the serum of suckling rats.

Refractive Index of Blood Serum in the Two Sexes.

In studying the general growth of the blood serum in respect to the refractive index, I did not at first make any attempt to separate the two sexes but mixed their bloods for this determination. This failure to separate the sexes was due in part to the fact that our preliminary test gave us an insignificant amount of difference between the two sexes and partly also because of the difficulty in obtaining enough blood serum, unless a considerable number of animals was used at one time. However, when the data were all accumulated and compared, it became very evident that there exists a distinct sex difference in the refractive index. To show this sex relation I have selected data from the original records in which the two sexes of the same age were separately determined, and the results are shown in Table IV.

As will be seen from Table IV there is no question that the refractive index of the serum differs in the two sexes. However, it was an unexpected outcome to find that the refractive index is slightly higher in the female than in the male, previous to the attainment of sexual maturity (85 days), while on the other hand it is decidedly lower after this stage. From the averages we find the following relations.

Average before 85 Days.

	N_D
Female	1.34736
Male	1.34606
Male	−0.00130

Average after 85 Days.

Male	1.35038
Female	1.34944
Male	+0.00094

TABLE IV.

Comparison of Data for Refractive Index of the Serum in the Two Sexes. Albino Rat.

Body weight.	Length.	Sex.	Age.	No.	Blood serum.				Remarks.
					Water.	Solids.	N_D	ΔN_D	
gm.	*mm.*		*days*		*per cent*	*per cent*			
14.4		♂	15	5	94.60	5.40	1.34487	11.67	Very cloudy.
21.3		♀	15	4	94.22	5.78	1.34504	11.84	16 hrs. after removal from mother.
24.8		♂	17	3	94.35	5.65	1.34538	12.18	16 hrs. after removal.
23.6		♀	17	4	94.11	5.89	1.34560	12.40	Cloudy.
27.9		♂	18	3	93.87	6.13	1.34632	13.12	Very cloudy.
25.9		♀	18	3	93.76	6.24	1.34632	13.12	" "
39.5	109	♂	23	4	94.35	5.65	1.34543	12.22	Clear.
38.0	105	♀	23	2	93.70	6.30	1.34598	12.78	Cloudy.
65.3	138	♂	36	1	93.76	6.24	1.34632	13.12	
62.6	129	♀	36	1	93.34	6.63	1.34692	13.72	
54.1	131	♂	37	2	94.12	5.88	1.34555	12.35	
51.3	126	♀	37	2	93.92	6.08	1.34606	12.86	
102.5	161	♂	66	4	92.78	7.22	1.34827	15.07	
79.9	149	♀	66	2	92.67	7.33	1.34841	15.21	
		♂	72	2			1.34863	15.43	
		♀	72	1			1.34872	15.52	
132.2	179	♂	79	1	92.56	7.44	1.34829	15.09	
123.2	172	♀	79	2	92.06	7.94	1.34898	15.78	
155.0	182	♂	81	2	92.36	7.64	1.34877	15.57	
118.7	167	♀	81	1	92.20	7.74	1.34872	15.52	
165.9	188	♂	85	2	92.88	7.12	1.34795	14.75	
119.5	176	♀	85	3	92.48	7 52	1.34889	15.69	
161.3	190	♂	93	1	91.87	8.13	1.35010	16.90	
119.5	170	♀	93	1	92.51	7.49	1.34898	15.78	Pregnant.

TABLE IV—*Concluded.*

Body weight.	Length.	Sex.	Age.	No.	Blood serum.				Remarks.
					Water.	Solids.	N_D	ΔN_D	
gm.	*mm.*		*days*		*per cent*	*per cent*			
127.4	175	♂	98	4	92.01	7.99	1.34980	16.60	Fasted for 48 hrs.
111.4	170	♀	91	4	92.24	7.76	1.34956	16.36	" " 48 "
177.7	192	♂	99	1	92.19	7.81	1.34941	16.21	
119.1	174	♀	99	1	92.58	7.42	1.34863	15.43	
353.5	238	♂	441	5	91.67	8.33	1 35014	16.94	Defective diet.
237.1	215	♀	471	4	92.37	7.63	1.34900	15.80	
255.7	211	♂	597	1			1.35245	19.25	
244.3	211	♀	597	1			1.35105	17.85	

Since there is no systematic change in the amount of the difference either in increasing or in decreasing according to age, shown in the table, we are justified in applying these corrections to the mixed data by either adding or subtracting the proper proportion of the differences given above from the mixed value in order to restore the proper sex values. I am unable to say whether or not a sex difference in the serum exists at birth, or for a few days after birth, and we must await further tests for the determination of this point.

Gettler and Baker (1916) have furnished the data on the refractive index of the normal human serum, which, though highly variable, show in the average values a slightly higher index in the male than in the female.[3]

	N_D
Average of 7 normal males (37 yrs.)	1.35061
" " 4 " females (42 ")	1.34998
Male	+0.00063

As is shown in Table IV the water content of the serum also shows a slight but correlated difference according to sex. The

[3] Positive Wassermann and doubtful cases were excluded. In the case of males the last seven cases were taken in order to make their average age as near as possible to those of the females.

water content of the male is slightly higher than that of the female before sexual maturity, and lower afterwards. From the average we find the following relation.

Average before 85 Days.

	Water. per cent
Male	93.58
Female	93.35
Male	+ 0.23

Average after 85 Days.

Female	92.44
Male	92.12
Male	− 0.32

In connection with the difference according to sex I may cite the work of Jones (1887) on the specific gravity of the entire blood of man and woman at different ages. It is evident that this value for the entire blood is complicated by the presence of blood corpuscles, and thus the entire blood cannot be compared directly with the serum alone. However the specific gravity determined by Jones shows most clearly a sex difference in this character.

The difference appears clearly at the time of puberty when the specific gravity becomes considerably higher in the male than in the female, and remains so for several years beyond 60. After the 69th year the data given by Jones show a reverse relation in the two sexes.

The specific gravity of the entire blood during the prepubertal period is, so far as can be judged by the data given by Jones, so fluctuating that it is difficult to interpret, though there is a strong tendency to a lower specific gravity in the female than in the male. This however may be due entirely to the greater relative abundance of the blood corpuscles in the male, so that these data do not help us to form any definite opinion concerning the relative amount of water in the serum of the two sexes.

Influence of Retardation or Acceleration of Growth on the Refractive Index of Serum.

Within a given litter of rats we often find that individuals show considerable difference in their power of growth. This dif-

ference is frequently so great that when the two extremes are compared the body weight of the best grown rat is two or even three times that of the smallest one. Since we found that the refractive index of the blood serum of the normally grown rat is a function of age, it was thought interesting to determine the indices in the serum of rats of the same age but of unequal size. The number of rats examined for this purpose is not large but the results obtained are striking.

TABLE V.

Refractive Index of the Serum of Rats of the Same Age but of Unequal Body Growth.

Body weight.	Length.	Sex.	Age.	No.	Blood serum.				Remarks.
					Water.	Solids.	N_D	ΔN_D	
gm.	*mm.*		*days*		*per cent*	*per cent*			
12.8	—	Mixed.	28	6	94.39	5.61	1.34504	11.84	Malnutrition.
42.6	115	"	28	4	93.59	6.41	1.34628	13.08	Normally grown rats. Different litters.
36.4	108	"	32	3	—	—	1.34479	11.59	Belongs to a litter of 12 rats.
—	—	—	32	—	—	—	1.34568	12.48	Estimated value of N_D for rats 32 days old.
38.7	116	♂	44	1	—	—	1.34445	11.25	Same litter.
82.1	149	♂	44	1	—	—	1.34649	13.29	
55.6	132	♀	69	1	—	—	1.34684	13.64	" "
182.0	190	♂	69	1	—	—	1.35010	16.90	

Table V shows that the undersized rats possess a serum which shows a much smaller refractive index than that of the better grown brothers or sisters. This point should be kept in mind when the normality of the refractive index of the serum comes up for consideration in any case.

Influence of Fasting, Defective Diet, and Gonadectomy on the Refractive Index of Blood Serum.

During the course of the investigation I have examined the serum of rats under several experimental conditions and I wish

to present the results, though the number of observations is rather limited.

Fasting.—Eight rats in one litter were divided into two groups, each consisting of two males and two females. These were deprived of food for 48 hours and at the end of this period one group was examined for the determination of the refractive index of serum, while the other four rats were fed with the normal laboratory diet and the blood was taken 2 hours later. It will be seen from Table VI that the fasting serum gives a distinctly higher index than that of the fed rats. Thus we may conclude that mild fasting tends to increase the refractive index of the serum. This high index may be due either to a slight reduction in the water content in the serum or to some other alteration in the constitution of the serum. It is known that as the result of fasting blood shows slightly increased amounts of solids (von Voit, 1881), an increase of specific gravity (Popel, 1896) and also an increase of blood corpuscles and hemoglobin content (von Limbeck, 1892). In this connection it is interesting to note that Robertson (1913) found an increase of the total serum protein in slightly fasted rats, and furthermore that mild fasting leads to an increase in the proportion of globulins to albumins in the serum of the rats. Since it has been shown by Reiss (1915) and by Robertson (1912) that the refractive index of serum globulin is slightly higher than that of serum albumin, and since fasting leads to an increase of the globulins, we may consider that the slightly higher refractive index following fasting might also be due to the increase of globulins. Robertson's rats showed this fasting effect only after 36 hours, without food.

Defective Diet.—For the opportunity to examine the blood serum of these rats I am indebted to Professors Osborne and Mendel. These rats were fed on diet adequate in all respects, except that the water-soluble vitamine has been added in very limited quantities, but sufficient for apparently normal growth and development. The rats thus raised, as Table VI shows, are practically normal to all appearance, but they are completely sterile. The weight of the testes was much diminished and spermatozoa were absent. The ovaries were large but a histological examination of them has not yet been made. The uterus of these rats showed an unusual yellow color indicating

the presence of some fatty degeneration or other pathological alteration.

As will be seen from Table VI, the refractive index is much lower than that of the normal rats of their age. This applies to

TABLE VI.

Influence of Fasting, Defective Diet, and Gonadectomy on the Refractive Index of the Serum.

Body weight.	Length.	Sex.	Age.	No	Blood serum.				Remarks.
					Water.	Solids.	N_D	ΔN_D	
gm.	*mm.*		*days*		*per cent*	*per cent*			
155.0	184	♂	85	4	92.37	7.63	1.34881	15.61	Control.
155.8	186	♂	85	4	92.95	8.05	1.34941	16.21	Castrated.
121.0	172	♀	85	4	92.24	7.76	1.34883	15.63	Control.
133.3	175	♀	85	4	92.32	7.68	1.34881	15.61	Spayed.
125.7	175	2♂+2♀	98	4	92.25	7.75	1.34950	16.30	48 hrs. fasting then 2 hrs. feeding.
113.1	170	"	98	4	92.00	8.00	1.34987	16.67	Fasted 48 hrs.
221.3	208	♀	435	1	92.52	7.48	1.34863	15.43	Raised by Osborne and Mendel with defective diet.
233.9	222	♀	438	1	92.27	7.73	1.34915	15.95	
225.4	205	♀	488	1	92.25	7.76	1.34924	16.04	
267.6	225	♀	524	1	92.44	7.56	1.34898	15.78	Both males and females sterile.
237.1	215		471		92.37	7.73	1.34900	15.80	Average.
405.7	249	♂	413	1	91.57	8.43	1.35028	17.08	
305.1	235	♂	418	1	92.04	7.96	1.34941	16.21	Infected lungs.
279.5	225	♂	421	1	91.66	8.34	1.35028	17.08	Slightly infected lungs.
407.1	239	♂	434	1	91.29	8.71	1.35088	17.68	
369.9	241	♂	519	1	91.79	8.21	1.34985	16.65	
353.5	238		441		91.67	8.33	1.35014	16.94	Average.
301.7	228		455		91.98	8.02	1.34963	16.43	Total average.

both the males and females. On the average these rats give the value of $N_D = 1.34963$ at 455 days, while the normally grown albino rat at the same age should give $N_D = 1.35122$. We therefore conclude that these rats possessed a serum which is clearly

different from that of the normals in respect to the refractive index, although their body growth, as well as their general health, was normal.

Gonadectomy.—The blood serum of castrated and spayed rats was examined for the refractive index. The testes or ovaries of these rats had been removed at the age of 39 days, and the blood was examined 55 days after operation, at which time the rats had of course completely recovered from the operation, and they were growing as well as their brothers and sisters.

The results show that the refractive index of the serum of castrated rats was distinctly higher than that of their uncastrated brothers. On the other hand the spayed rats give a slightly low refractive index when compared with their normal sisters. I am unable to account for this difference in reaction according to sex, or even to determine whether this difference is really significant. Biedl (1913) states the effect of castration on the blood in the following terms: "Während Pinzani bei Kastration Hündinnen eine Zunahme des Hämoglobins und der roten Blutkörperchen gefundene haben will, sah Lüthje keine Veränderungen dieser Bestandtheile." I have failed to find so far any other references in connection with this question and therefore present the data on the rat without further comment.

Refractive Index of Blood Serum of the Norway rat (Mus norvegicus).

A careful investigation of the blood serum of the Norway rat is highly desirable, but is not easy to make. The chief difficulty is to rear the Norway rat without producing a domestication effect, while from the trapped rats we cannot get data which are important for the treatment of the refract ve index. A few observations on the wild Norway rats have been made and these data are presented for comparison with those from the albino (Table VII).

If the usual assumption is made that difference in body length generally coincides with a difference in age, we may conclude that the refractive index of the serum of the Norway rat also would increase with age, as in the case of the albino rat. The value of the index is however slightly smaller than that given by albino rats of the same body length. The Norway rat in which the body

length is 205 mm. gives 1.34874 for the refractive index, while the albino rat having the same body length, gives 1.34980, or a difference of as much as 0.00106 in favor of the albino rat. However, it is known that for the same body length, the Norway rat is probably younger than the albino; therefore if we are able to make age correction for the above values, the refractive indices of the serum in these two forms might come closer together.

TABLE VII.

Refractive Index of Blood Serum of the Wild Norway Rats of Different Body Lengths.

Body length.	Sex.	No.	Blood serum.		Remarks.
			N_D	ΔN_D	
mm.					
136	Mixed.	2	1.34658	13.38	
159	♀	1	1.34726	14.06	
194	♂	1	1.34872	15.52	
240	Mixed.	2	1.34985	16.65	
243	♂	1	1.35019	16.99	Slight hemolysis.
256	♂	1	1.34985	16.65	
Average . . . 205			1.34874		

General Remarks.

From the data already presented we notice that the refractive index of the serum changes not only with age but according to sex. We also have found that the refractive index of the serum taken from rats suffering from lung infection, raised on a defective diet, fasted for 48 hours, or those the growth of which had been retarded, etc., shows considerable deviations from that taken from the normal rat. This alteration in the refractive index is closely related to the water content of the serum, and in general the higher the refractive index the lower is the water content of the serum. There is also evidence of a change in the relative protein content of the serum. Therefore changes in the refractive index mean also changes in the relative degree of concentration, and it thus follows that the colloidal state of the serum is also altered. It is probable that these changes bear directly on the

phenomena of agglutination and the precipitin reaction, but these relations are still to be tested. Furthermore, it seems to me that on account of its extreme simplicity, yet high degree of sensitivity, the refractometer test might advantageously be applied to the study of other body fluids.

CONCLUSIONS.

1. The refractive index of the blood serum changes according to the age of the rat, and in general it increases with increasing age. The data form a good growth curve. From the course of the change in the refractive index we may conveniently divide the entire life span into three periods: (1) From birth to the end of the suckling period. Before suckling the refractive index of the serum is higher than after suckling. However, soon after suckling the refractive index falls and then rises rapidly until near the end of the suckling period (18 days). The first partial intake of solid food brings the serum index promptly down, and the lower values continue till the rat changes entirely from a milk to a mixed diet (about 23 days of age). (2) The period of sexual maturity. Soon after the rat has become adjusted to the change of diet, the refractive index rises again steadily until towards the attainment of sexual maturity (70 days old). Following this rise is a period of 2 weeks in which the index is nearly stationary. (3) Adult period. After 90 days in age the refractive index of serum slowly rises throughout the remainder of the life span, so far as examined (up to 600 days).

2. The water content of the serum also varies with age. It is highest at birth and becomes lower with increasing age. The course of alteration in the water content is closely related to the alteration in the refractive index, only it is inverse.

3. Up to 85 days the refractive index of the serum is slightly higher in the female than in the male. This relation is reversed after 85 days of age, when the male serum gives a considerably higher index than the female serum.

4. The retardation of growth gives a lower refractive index, while over grown rats give higher indices when compared with average normal rats.

5. Fasting (48 hours) produces a slight increase in the refractive index, while a defective diet (small amount of water-soluble vitamine) produces a slight reduction in the index.

6. The refractive index of the serum in the Norway rat varies regularly and increases with increasing body length.

7. The value of the refractive index of the human serum agrees with that of the albino rat on the following points: (1) Absolute value of N_D of the serum is practically the same both in man and in the rat at the adult stage. (2) The refractive index of the serum is higher before suckling than after suckling, and furthermore the refractive index rises rapidly after this initial fall, as in the case of the albino rat. (3) From birth to puberty, however, the values for man are higher than those for the albino rat.

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A STUDY OF THE CARBON DIOXIDE-COMBINING POWER OF THE BLOOD PLASMA IN EXPERIMENTAL TETANY.*

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Recent investigations of the acid-base equilibria in pathological conditions have been slowly adding to our knowledge of the factors involved in their maintenance. Acidosis seems first to have caught the attention of investigators, probably because of the greater frequency with which it is encountered. Recent studies by Wilson, Stearns, and Janney (1), Wilson, Stearns, and Thurlow (2) have directed attention to the condition of alkalosis. These investigators have found that in dogs following parathyroidectomy the equilibrium between acids and bases is displaced in favor of the bases, and that in tetany which develops after such a procedure there is well marked alkalosis. This was developed in a study of the hemoglobin dissociation, and of the alveolar air in dogs following thyroparathyroidectomy.

From an uncompleted series of experiments on dogs, undertaken to study the gastric secretion in tetany, the following data regarding the carbon dioxide-combining power of the plasma are taken. These data give confirmation to the conclusions of Wilson and coworkers with regard to the results following parathyroidectomy. Furthermore, studies were made on dogs in which tetany was induced by various operative procedures on the stomach. In these latter cases changes in the acid-base equilibrium were noted similar to those following parathyroidectomy.

The method of investigation chosen was simple. The ability of the plasma to combine carbon dioxide at alveolar tension, deter-

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mined by the method of Van Slyke and Cullen (3), was taken as the measure of the alkali reserve. This was chosen because of its greater simplicity than the hemoglobin dissociation method, and in place of the examination of the alveolar air, because the carbon dioxide content of the latter is affected by the changes in the irritability of the respiratory center which occur in tetany. The method of Van Slyke is not open to this latter objection.

The studies were carried out on dogs. Parathyroidectomy without thyroidectomy was done on two dogs. Five other dogs were subjected to gastric operations. In one the pylorus was ligated, another developed tetany with an hour-glass constriction following Pawlow's operation. In three other dogs the stomach was divided just on the proximal side of the pyloric sphincter. After drawing the cut ends between the split fibers of the rectus muscles, they were sutured to the skin, thus making two openings, one into the stomach, the other into the duodenum through the pyloric sphincter. It was hoped that by frequent emptying of the stomach the secretion could be obtained entire. The results were rather disappointing, except in one case in which the full data regarding the gastric secretion are given. These are too meager to justify any conclusions. All of the dogs developed tetany, without any infectious complications, and in all of the cases satisfactory data regarding the carbon dioxide-combining power were obtained.

Blood for examination was drawn from a heel vein, oxalated, centrifuged, and the plasma stored in a paraffined tube on ice unless the examination could be made immediately. The apparatus used was that of Van Slyke. The plasma was saturated with air containing 6 per cent by volume of carbon dioxide, previously mixed in a well calibrated spirometer. The only other variation from the usual technique was the substitution of *n*-heptyl alcohol for the caprylic alcohol used by Van Slyke.

All operative procedures were carried out with strict aseptic precautions, under ether anesthesia administered intratracheally.

Protocols.

Dog 48.—Dec. 11, 1916. Parathyroidectomy under intratracheal ether anesthesia. Removed the parathyroids on both sides without removing the thyroid. Verified by section. Blood taken before operation.

Dec. 12. Condition good, lively, not vomiting.

Dec. 13, 9 a.m. When first seen was in violent tetany, exhibiting the usual symptoms; contractures of the fore legs, twitching and jerking, hyperpnea, and profuse salivation.

Hrs. after Operation.	*CO_2 Combined.* vol. per cent
Before	52.0
24	63.0
48	79.4
48½	74.3

At 10.10 a.m., a molecular solution of magnesium sulfate was injected under the skin, dose equal 0.27 gm. per kilo of body weight. Within half an hour the relaxation was well advanced. The respirations decreased from 280 to 76 per minute. At 12.30, the respiration was 17. The dog had a few slight spasms from time to time. A 0.02 M solution of $CaCl_2$ was injected intravenously at 12.40 p.m. This induced severe tetanic spasms after a dose of 0.004 gm. per kilo of body weight. At 1.40, the dog had a severe convulsion and died.

Dog 11.—Mar. 26, 1917. Two parathyroids were removed from the posterior aspect of the left lobe of the thyroid gland. On the right side they could not be identified, so that the right thyroid lobe was removed entire. At intervals the stomach contents were removed through a tube, and blood was taken for determination of the CO_2-combining power.

Hrs. after Operation.	*CO_2 Combined.* vol. per cent	*Free HCl, 0.1 N.* cc.	*Total Acid, 0.1 N.* cc.	*Remarks.*
Before	47.5			
24	48.3	73.5	194.8	Lively.
43	65.4	25.0	37.5	Condition good.
77	78.6	0	0	Twitching, spastic fore legs.

Dog died during the night, apparently of tetany. Necropsy showed no other cause of death except tetany.

Dog 2.—Feb. 7, 1917. Large black and white male dog. Blood taken Feb. 6; plasma combines 59.3 volume per cent CO_2. Operation: ether anesthesia; ligation proximal to pylorus with heavy braided silk ligature. Blood withdrawn at intervals, and CO_2-combining power determined (see tabulation below). Water given by catheter into the rectum. Condition of animal remained excellent until Feb. 10, when muscular twitching commenced. During the preceding 24 hours the dog had been drinking considerable water and vomiting. Mild symptoms of tetany continued during the next 24 hours, and on the night of Feb. 11 severe tetany developed. The animal was much improved on the following morning, though the CO_2-combining power was so great that it could not be determined accurately in the Van Slyke apparatus, the reading being 1.2 cc. of CO_2 per 1 cc.

of plasma. A gastroenterostomy was performed by the anastomosis of a lower loop of jejunum to the anterior wall of the stomach, Feb. 12. During the next 24 hours no tetany was observed; the blood drawn combined only 50.4 volume per cent of CO_2. However, the interpretation of this drop is vitiated by the fact that the dog developed a pneumonia. He was killed with ether the following day. Autopsy showed early pneumonia in the left lower lobe. The stomach was being drained by the gastroenterostomy; peritoneum and wounds uninfected.

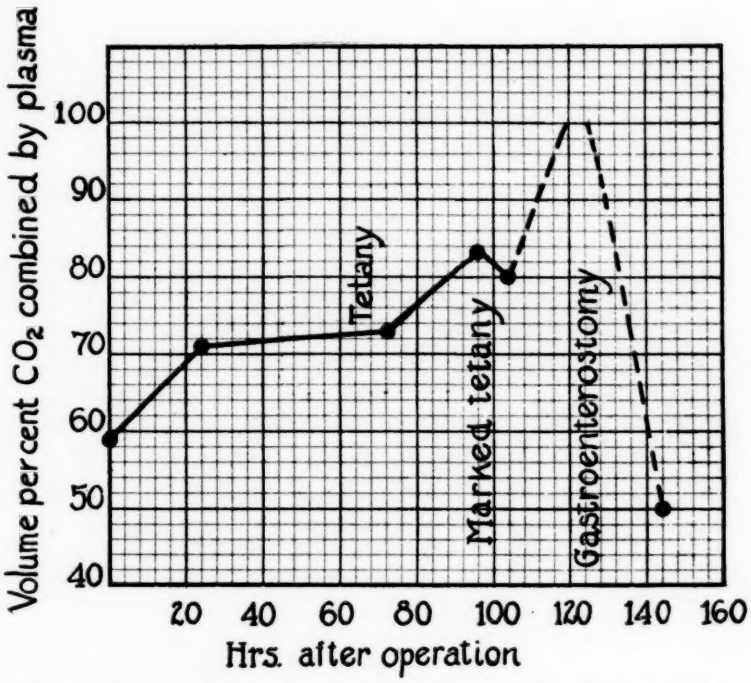

FIG. 1. Dog 2. Pyloric ligation with subsequent gastroenterostomy.

Hrs. after Operation.	*CO_2 Combined. vol. per cent*
Before	59.3
24	70.6
72	72.5
96	83.5
104	80.0
120	Too high to read.

Dog 7.—Rather old, black male dog; weight, 11 kilos. Operated 10 a.m., Feb. 20, 1917, for the production of a Pawlow stomach. 12 hours later the dog vomited and drank a great deal. Blood plasma at this time combined 58.7 volume per cent of CO_2. On the following day mild tetany was observed, evidenced by shivering and contractures of the fore legs. Plasma combined 64.4 volume per cent of carbon dioxide. At this point an injection of 0.33 N HCl was made into a heel vein until the respiration rose from 20 to 46 per minute. A specimen of plasma taken 30 minutes later showed a combining power of 46.7 volume per cent. No further evidence of tetany was seen following this until 4 p.m., Feb. 22, when the dog had violent tetanic seizures. The CO_2-combining power was found to be 79.1 volume per cent. The dog died during the night. Autopsy showed the presence of an hour-glass constriction of the stomach, with no other cause of death assignable.

Hrs. after Operation.	*CO_2 Combined.* vol. per cent
Before	No data.
12	58.7
24	64.4
24½	46.7
40	79.1

Dog 19.—Brown bitch, short hair; weight 12 kilos. Apr. 3, 1917. Operation: division of the pyloric end of stomach, with formation of two stomas, one into the distal, and one in the proximal end of gut. Rectus muscles split to form sphincters. Just before the operation the CO_2-combining power of the plasma was 49.2 volume per cent. As the sphincter action of the muscles was imperfect, it was not possible to obtain all the gastric secretions.

Hrs. after Operation.	*CO_2 Combined.* vol. per cent
Before	49.2
6	71.6
23	81.1
30	82.2

23 hours after operation the animal began to have contractures of the fore legs and marked salivation. At the end of 30 hours it went into such violent tetanic spasms that it was killed after withdrawing blood for the determination of CO_2-combining power.

Dog 17.—Mar. 19, 1917. White, shaggy female; weight 12 kilos. Blood drawn before operation combined 48.3 volume per cent of CO_2. Ether anesthesia. Operation: stomach divided near the pylorus after ligating the vessel on either curvature. The cut ends received a lock stitch for hemostasis, after which they were drawn through the split fibers of the rectus muscles. Each end was then sutured to the fascia and skin mak-

ing two fistulas, from which gastric or duodenal contents did not escape. The stomach was washed at frequent intervals by means of a catheter inserted through the gastric fistula. The washings were measured, titrated for free HCl and total acidity, and the results expressed as the total number of cc. of 0.1 N acid secreted. These results are given in tabular

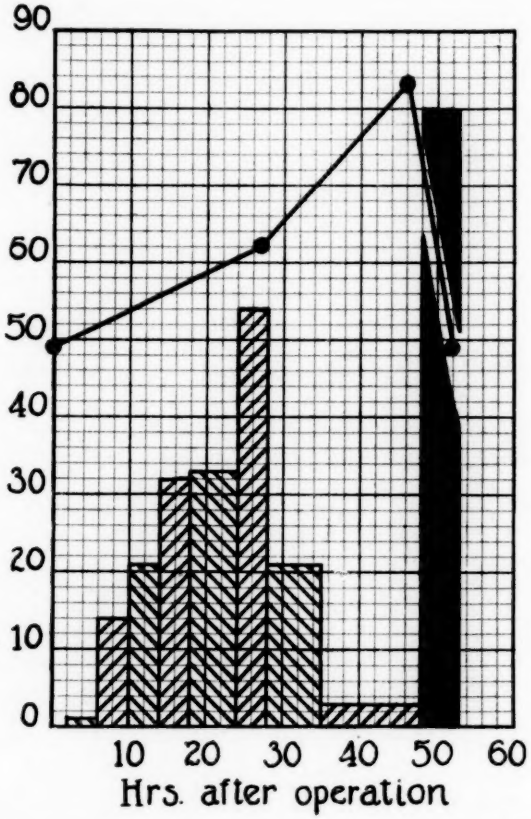

FIG. 2. Dog 17. The curve represents the volume per cent of CO_2 per hour combined by plasma. The cross-hatching signifies cc. of 0.1 N acid secreted; the solid black, cc. of 0.1 N acid injected.

form below. Water was given to the animal through another catheter which was inserted through the pyloric sphincter into the duodenum. The animal drank very little water and did not vomit.

Mar. 20. Condition of dog good, and no symptoms of tetany observed. On the following day tetany began with tremors and spasmodic twitch-

ings, which developed in the course of 2 hours into severe seizures, in which the animal lay on its side with legs stiffly extended and twitching. Salivation marked. Respiration very irregular in rate, ranging from 45 to 180 per minute. A large amount of alkaline urine was passed, and a loose watery stool. Blood combined 83.7 volume per cent of CO_2. After withdrawing blood 400 cc. of 0.4 per cent HCl were injected through a catheter into the duodenum. An hour later the dog was walking about. After 4½ hours the animal had a seizure of tetany which lasted 5 minutes. Blood was drawn and found to combine 49.2 volume per cent of CO_2. The dog was then killed.

Autopsy performed at once. Heart, lungs, liver, spleen, and kidneys were normal. The wounds were clean, there was no intraperitoneal infection. The upper part of the small intestine was filled with a large amount of alkaline bile-stained fluid. The stomach was empty.

CO_2-Combining Power of Plasma.

	vol. per cent
Mar. 19, 10 a.m.	48.3
" 20, 2 p.m.	61.6
" 21, 9 a.m.	83.7
" 21, 2 p.m.	49.2

Amounts of Acid Secreted by Periods.

Period.	No. of hrs.	Total cc. 0.1 N secreted.	Rate, cc. 0.1 N per hr.
1	4	2.72	0.68
2	4	56.44	14.11
3	4	85.68	21.42
4	4	122.40	30.60
5	6	203.00	33.83
6	4	216.00	54.00
7	6.5	130.50	20.08
8	13.5	49.00	3.65

Dog 18.—Mar. 28, 1917. Division of pyloric antrum. Ends drawn through the rectus muscle. Blood drawn before operation, and periodically thereafter. Mar. 29. Dog in good condition. The sphincter action of the rectus muscles is imperfect. Mar. 30. Dog developed tetany. The blood drawn 1 hour before combined 81.2 volume per cent of CO_2. 400 cc. of 0.4 per cent HCl solution were introduced through the pyloric sphincter into the duodenum at 12 noon. At 4 p.m., symptoms of tetany had disappeared, and the CO_2-combining power was found to be 56.1 per cent. The dog remained free from tetany until the following day, when the CO_2-combining power was 53.5 per cent. The dog was then killed.

Hrs. after Operation.	*CO_2 Combined. vol. per cent*
Before	57.2
24	65.9
29½	74.5
48	81.2
52	56.1
72	53.5

The gastric juice could not be collected quantitatively, but three samples taken gave the following figures (cc. 0.1 N acid per 100 cc. of gastric contents).

Mar. 29, 11 a.m.	Free HCl	102	Total acid	130
" 29, 4.30 p.m.	" HCl	80	" "	125
" 30, 4 p.m.	No acidity.			

These figures show that during the period when the CO_2-combining power was increasing acid was being secreted.

DISCUSSION.

The foregoing experiments show consistently an increase in the ability of the plasma to combine carbon dioxide coincidently with the development of tetany. This may be interpreted as being due to an increase of the basic over the fixed acid radicles in the blood, as compared with that of normal animals.

These experiments, with the exception of one, cast little light on the mechanism involved in the displacement of the acid-base equilibrium. The mechanism for the maintenance of this equilibrium is very complex. Recent studies of the acid-base excretion in nephritis have called attention to the importance of changes in the permeability of the kidney to acid or basic phosphates. Fitz, Alsberg, and Henderson (4) observed the increased phosphate excretion in experimental acidosis. Marriott and Howland (5) observed an acid-phosphate retention in certain cases of acidosis in chronic nephritis. However, Greenwald (6) finds a decreased phosphate excretion after parathyroidectomy, which is inconsistent with the view that tetany is the symptom complex of alkalosis unless the phosphates retained were alkaline, the equilibrium being shifted so that the concentration of dihydrogen phosphates is diminished.

The effect of antagonistic electrolytes, on both irritability and permeability, must be considered. Osterhout (7) has made a review of the relation of permeability and antagonism. McClendon (8) presents some interesting observations on the relation between certain antagonists and irritability. The pulsation rate of the jellyfish, *Cassiopeia xamachana*, is increased by increasing the concentration of OH, Na, or K ions. It is decreased by the H ion, by Mg and Ca ions. A similar antagonism was observed between the same ions in their effect upon the heart of the conch *Strombus gigas*. That there is an increased irritability in tetany is shown by a study of the electrical reactions made by MacCallum and Voegtlin (9), who have also noted a diminution of calcium in the blood and an increased excretion of calcium. The relief of tetany by the injection of metals of the alkaline earth group (Mg and Ca) has been observed by various writers (1), especially Voegtlin and MacCallum (10). Wilson, Stearns, and Janney quote this and add (1) that the injection of the alkali metals (such as Na and K) increases the severity of tetany, and that acids injected intravenously relieve it. The effect of the administration of acid both intravenously and through the duodenum is strikingly shown in the cases of Dogs 7 and 17.

The work of Henderson and Haggard (11), published since the completion of this paper, makes it necessary to consider another possibility; namely, that through some means the irritability of the respiratory center is increased causing decreased ventilation and a secondary change in the CO_2-combining power. Although no special effort was made to determine this, there was no obvious change in respiration on the part of these dogs, and in every case a considerable increase of CO_2-combining power occurred before any clinical evidence of tetany. The results obtained by McClendon, as quoted above, would also make it seem probable that the plasma change preceded the irritability of the respiratory and other nerve centers.

One remaining factor deserved consideration as entering into the maintenance of acid-base equilibrium,—the secretion of acid by the stomach. Clinically gastric tetany is most apt to occur in those cases in which there is some pyloric obstruction. The secretion of hydrochloric acid by the stomach, provided the acid does not pass into the duodenum to excite the alkaline intestinal secre-

tions, might be expected to leave the blood with an excess of basic substances. The so called alkaline tide, following a meal, may be due to this. The evidence on this point is in no way conclusive. In the case of Dog 2 the effect of a gastroenterostomy in producing a marked and prompt drop in the CO_2-combining power is suggestive in indicating that possibly the balance between acid and alkali secretion was reestablished by the stimulus of the gastric acid in the duodenum. In the case of Dog 17 the rate of acid secretion is seen to have increased during the period in which the CO_2-combining power was increasing, until this passed the upper normal limits. In this same dog the effect of introducing acid into the duodenum is out of proportion to the amount introduced, the probable explanation being that the blood alkalies were not only being neutralized but were also being lost through secretion. Dog 18 also showed a marked and lasting beneficial effect after the introduction of acid into the duodenum, in contrast to the transient effect from intravenous injection (Dog 7).

CONCLUSIONS.

These experiments having been interrupted before reaching a point at which definite conclusions could be drawn, they seem nevertheless to indicate the following:

1. After parathyroidectomy there is a marked increase in the carbon dioxide-combining power of the blood plasma, coincident with the development of tetany.

2. Following operations on the stomach, which exclude the acid secreted from the duodenum, tetany develops accompanied by an increase in the carbon dioxide-combining power of the plasma similar to that of parathyroid tetany.

3. Tetany is a condition of alkalosis, in which a disproportion between the rates of secretion of acids and alkalies by the gastrointestinal tract may be a factor.

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A MODIFIED METHOD FOR THE PREPARATION OF PICRAMIC ACID.

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The importance of picramic acid as a standard for Benedict's blood sugar determination and the present shortage of picric acid suggested to the writer that the small yield of picramic acid obtained by working in accordance with the instructions in the literature might be improved.

One method commonly used for the preparation of picramic acid is that of Girard,[1] which states: If a cold saturated alcoholic solution of picric acid is neutralized with ammonium hydroxide and then saturated with hydrogen sulfide the liquid turns red and small red crystals are deposited. On distilling off the alcohol, sulfur and additional red crystals are deposited. They are the ammonium salt of picramic acid from which picramic acid can be obtained on addition of acetic acid to the hot aqueous solution.

The other method reported by Lea[2] reads as follows: An alcoholic solution of picric acid treated with an excess of ammonium sulfide is evaporated on the water bath, the residue extracted with boiling water, and in the filtrate the picramic acid is precipitated with acetic acid. 63 per cent is claimed as yield. The writer was unable to confirm this last statement.

As to Girard's methods, if one follows the instructions literally, that is, if one uses a cold saturated solution of picric acid neutralized with ammonium hydroxide, the larger proportion of the crystals deposited on passing hydrogen sulfide into the solution consists of unchanged picric acid. It takes at least from 1 to 1½ hours to deposit any crystals of the reduction product and these are greatly contaminated with the diamino salt due to the heat resulting from reduction. The product obtained when using Lea's method is also contaminated to a great extent with the diamino salt.

[1] Girard, A., *Compt. rend. Acad.*, 1853, xxxvi, 421; *Ann. Pharm.*, 1853, lxxxviii, 281.

[2] Lea, C., *Jahresbr. Fortschr. Chem.*, 1861, 637; *Am. J. Sc. and Arts*, 1861, xxxi, 188; xxxii, 210; *Chem. News*, 1861, iv, 193; *J. prakt. Chem.*, 1862, lxxxvi, 319.

Picric acid remains in solution in the presence of an excess of ammonium hydroxide, and the formation of the ammonium salt of the diamino-acid is almost excluded by cooling the solution.

The following modification of the procedure proved satisfactory.

20 gm. of finely powdered picric acid are heated in 300 cc. of alcohol in an Erlenmeyer flask of 600 cc. capacity (boiling the solution should, however, be avoided so as not to decrease unnecessarily the quantity of liquid). When dissolved, 125 cc. of concentrated ammonium hydroxide are added and the contents of the beaker cooled to 30°C. with running water. A very rapid stream of washed hydrogen sulfide gas is passed into the solution until the thermometer shows a temperature of 50–55°C. This temperature is reached in about 10 or 15 minutes, depending on the rapidity with which hydrogen sulfide is passed in, and should not be exceeded. Without discontinuing the stream of hydrogen sulfide the flask is placed in crushed ice and hydrogen sulfide allowed to pass through for 45 minutes more. The delivery tube should be at least 1 cm. wide.

The crystals of the ammonium salt of picramic acid which have now been deposited in large quantities are freed from all mother liquor by filtration on a Buchner funnel, transferred to a porcelain dish, and treated with 60 cc. of acetic acid (1 part of glacial acetic acid to 4 parts of water) to decompose the ammonium salt. The mixture is vigorously stirred so as to bring all solid substance into reaction with the acid. The picramic acid is filtered off on a Buchner funnel and washed with two portions of water, using 10 cc. each time. The yield should be from 14 to 15 gm. The acid is purified as follows: 500 cc. of distilled water are heated to boiling, and 2 gm. of the crude picramic acid are added and boiled in the water with stirring. Not more than 50 cc. of the water should be allowed to evaporate. The undissolved part, consisting chiefly of sulfur and a trace of diamino-acid, is filtered off through a folded filter. The filtrate is allowed to cool in an ice box or in ice to a temperature of from 10–12°. The picramic acid is filtered off by suction and air dried.

The picramic acid thus obtained has a melting point of 168°C. as required of the pure acid. The yield from 20 gm. of picric acid is 13.3 gm., which is 76.5 per cent of the theoretical value.

If the residue, obtained by concentration of the alcoholic filtrate from the ammonium picramate, is treated with 500 cc. of boiling water, filtered, and acidified with 5 cc. of glacial acetic acid, cooled with ice to about 10–12°C., an additional yield of picramic acid is obtained. It is filtered on a small Buchner funnel, washed with two portions of water of 5 cc. each. The yield is increased about 5 per cent of the theoretical value.

The writer wishes to express appreciation for assistance in the experimental work to Miss Frances Ford.

THE METABOLISM OF GLYCINE GIVEN INTRAVENOUSLY AT CONSTANT RATES.

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(Received for publication, July 22, 1918.)

For a long time the progress of our knowledge of the fate of ingested protein was halted because of the inability to trace it further than the lumen of the intestines. We were certain that it was digested by enzymes more or less completely to the amino-acid stage, but further than this we were unable to go because of the lack of methods for detecting amino-acids in the blood or of identifying possible products of the ingested protein after leaving the intestines. As a result there were formulated a number of hypotheses to explain the method by which the body received into its substance the protein of the food. All these hypotheses had arguments in their favor but each lacked decisive data which proved it. It was not until Van Slyke devised a method for determining small amounts of α-amino-acids that we were fairly certain that proteins were absorbed into the blood stream as amino-acids. By this method the distribution and fate of these absorbed amino-acids can be followed. It is shown that they are quickly removed from the blood stream by the tissues, especially the liver and muscles, and in these tissues further steps in their metabolism are carried on. The first of these steps appears to be the splitting off of the NH_2 from the carbon moiety, the process of deaminization. Van Slyke shows that this occurs at a much slower rate than that at which the amino-acids are removed by the tissues from the blood stream. The NH_2 group is eliminated in the urine as urea and ammonia. The deaminized portion ultimately is oxidized to CO_2 and water. A certain part of the amino-acid is no doubt used for the synthesis of new body proteins.

Since we are now fairly well acquainted with the qualitative history of the amino-acids, refinements of study can be introduced which involve a quantitative aspect. Certain viewpoints which have been developed in the study of carbohydrate metabolism can now be applied to amino-acids. The most recent idea is that of Woodyatt who introduces the factor of rate in the study of intermediate sugar metabolism in the attempt to interpret the biological processes concerned in terms of quantitative chemistry. His method is to introduce sugar or other soluble metabolites into the circulation at known and constant rates for considerable periods of time until equilibria are reached. A study of the rates of excretion and other indices of metabolic processes under these conditions will give data concerning the amount and manner of utilization which can be discussed from the view-point of reaction velocities. In order to maintain a constant rate of entry into the blood stream and to avoid the uncertainties of absorption times, Woodyatt devised an electrically driven machine which delivers measured amounts of fluid directly into the blood stream, accurately adjusted at so much per unit time. With this apparatus, intravenous injections can be kept up indefinitely and constantly at any desired rate. For a fuller discussion of the theory and method of timed intravenous injections the reader is referred to Woodyatt's articles.[1]

This paper is the first of a series in which the metabolism of various amino-acids will be studied in the same manner as Woodyatt *et al.* have studied sugars. They found in the case of glucose that this sugar can be injected for at least 9 hours at any rate up to and including 0.85 gm. per kilo of body weight per hour without causing glycosuria. Above this rate glucose regularly appears in the urine. The question which concerns us now is how rapidly any given amino-acid can be injected continuously without producing an overflow of unchanged amino-acid in the urine.

[1] Woodyatt, R. T., Studies on intermediate carbohydrate metabolism, *Harvey Lectures*, 1915–16, xi, 326; The method of timed intravenous injections, *J. Biol. Chem.*, 1917, xxix, 355. Woodyatt, R. T., and Sansum, W. D., Studies on the theory of diabetes. VIII. Timed intravenous injections of glucose at lower rates, *ibid.*, 1917, xxx, 155.

The amino-acid selected for study first is glycine, because it is the simplest amino-acid and is easily prepared in pure form. The sample used was prepared by Dr. E. J. Witzemann of this Institute by synthesis from monochloroacetic acid and ammonia. The product was of a high degree of purity, occurring as large colorless rhombic crystals (melting point 243–245°, corrected). The glycine was dissolved in 0.9 per cent sodium chloride solution at approximately the desired concentration. The exact concentration was determined by the Van Slyke amino-acid method. Dogs which had fasted for 24 hours were used. Before each experiment began, the bladder was washed out. There was always a period of 2 hours or more preceding the injection during which a sample of normal urine was obtained. In some of the experiments salt solution was injected before the glycine solution, in order to insure the hourly secretion of enough urine for analysis. Glycine was injected at rates varying from 0.1 to 1.5 gm. per kilo of body weight per hour. On all samples the following determinations were made: total nitrogen by the Kjeldahl method, ammonia nitrogen by the Folin aeration method, urea nitrogen by the Marshall urease method, and amino-acid nitrogen by the Van Slyke method. Comparisons of the effect of the injection of glycine were made with the basal excretion of nitrogen determined from the analysis of the urine of the fore period. Owing to the limitations of the method for determining amino-acid in the urine, together with the small quantities of urine excreted per hour by dogs weighing from 7 to 16 kilos under the conditions of the present experiments, it was not found feasible, except in certain cases, to follow accurately the hourly excretion of the several nitrogenous substances named above. The urine was therefore collected for periods of 2 to 6 hours or more. In the case of the more rapid glycine injections, toxic effects prohibited their being sustained for more than an hour. Accordingly equilibria as indicated by the attainment of constant ratios between the rate of nitrogen injection and the rate of nitrogen excretion were not established except in the case of the lower injection rates. But the short injections at the higher rates show roughly the relative quantities of nitrogen excreted in the form of unchanged glycine and as urea and ammonia, respectively, following injections at these definite rates.

It appears that the tolerance limit for glycine injected into the systemic venous blood of resting dogs is close to 0.2 gm. per kilo per hour. More experiments would be necessary to fix this value; *e.g.*, repeated experiments at 0.3, 0.2, and 0.1 gm. rates. But it is suggested that glycine entering the systemic blood stream at a rate not exceeding 0.2 gm. per kilo per hour may enter the tissues and be metabolized as fast as it is injected, hour after hour, while with faster rates of injection the metabolism of glycine fails to keep pace with the rate of supply and some glycine passes through the body unchanged. When this figure is compared with the tolerance limit for glucose (0.85 gm. per kilo per hour) it forms a basis for comparing the relative normal velocities of the glucose and glycine metabolism.

The data also show, in harmony with established facts concerning amino-acids in general, that their NH_2 groups are split off and enter into formation of urea even when the amino-acids are injected directly into the systemic blood stream. The excretion of this urea begins soon after injection but may drag along for 18 hours or more afterwards. The excretion of unchanged glycine, on the contrary, appears to be completed at a much more rapid rate, although far more slowly than that of sugar. In the latter case we are dealing with an excretion rate; in the former, the rate of metabolism and the rate of excretion both come into play. This suggests the possibility of measuring the velocity of deaminization by determining the rate of urea excretion after simple urea injection and after injection of amino-acids (subtracting one from the other).

Lusk[2] and others found that glycine is converted into glucose in the completely diabetic organism in the ratio of 0.8 gm. of glucose from 1 gm. of glycine. From this fact it has been held that the carbon residue of glycine after deaminization undergoes the same changes in the normal body. If glycine is thus converted into glucose in the normal non-diabetic body, and this conversion occurs at an absolute rate above 0.85 gm. of glucose per kilo of body weight per hour, it might be expected that a glycosuria would occur. It would require the metabolism of 1.06

[2] Lusk, G., Elements of the science of nutrition, Philadelphia, 3rd edition, 1917.

gm. of glycine per kilo of body weight per hour to introduce glucose into the metabolism at the rate of 0.85 gm. per kilo per hour on the basis of Lusk's conception. In the present experiments, glycosuria was regularly absent, but in none of them was there any evidence that the rate of glycine metabolism equalled 1.06 gm. per kilo per hour. These particular experiments therefore cast no light on this subject. At the highest injection rate, 1.5 gm. for 1 hour, we do not know how the material was metabolized and with the long slow injection we would not expect glycosuria. This question might be attacked successfully by a study of the rate of sugar excretion in the completely diabetic animal when glycine was injected at various rates.

It was interesting to note that in Experiment V, during the 3rd hour of injection the dog became very ill. It vomited, had diarrhea, and suffered from severe convulsions, very much resembling the symptoms one sees in Eck fistula dogs after feeding large amounts of meat. Just as soon as the injection was discontinued these symptoms disappeared.

SUMMARY.

1. The tolerance rate for resting dogs of glycine injected continuously into a systemic vein is probably close to 0.2 gm. per kilo of body weight per hour.

2. Glycine thus injected intravenously at constant rates is readily converted into urea.

3. The excretion of unchanged glycine is materially slower than that of sugars.

Experiment I.

Injection of Glycine at the Rate of 1.5 Gm. per Kilo of Body Weight per Hr. for 1 Hr.

Weight of dog, 7.22 kg. Injected 10.83 gm. of glycine, containing 2.03 gm. of N.

Period I = 6 hrs. Normal control. Before injection.
" II = 6 " Period during which glycine was injected for 1 hr.
" III = 12 " After period with no injection.

Analysis of Urine.

Period.	Hrs.	Total N.	Urea N.	Ammonia N.	Amino N.
		gm.	*gm.*	*gm.*	*gm.*
I	6	0.476	0.34	0.04	—
II	6	1.800	1.04	0.03	0.428
III	12	1.748	1.35	0.04	0.039

Amount of N injected as glycine, *gm*	8.03
" " N recovered " " *gm*	0.47
Injected glycine excreted unchanged, *per cent*	23.1
Amount of extra N in urine resulting from injection (total), *gm*	2.12
Injected N recovered in urine, *per cent*	100

It would appear that the excretion of glycine had almost or quite come to an end and that the excretion of urea had reached the basal level at the close of the After Period III, while the ammonia excretion remained unaffected by the injection.

Experiment II.

Injection of Glycine at the Rate of 1.13 Gm. per Kilo of Body Weight per Hr. for 1 Hr.

Weight of dog, 12.5 kg. Injected 14.13 gm. of glycine, containing 2.64 gm. of N.

Period I = 6 hrs. Normal control. Before injection.
" II = 6 " Period during which glycine was injected for 1 hr.
" III = 12 " After period with no injection.

Analysis of Urine.

Period.	Hrs.	Total N.	Urea N.	Ammonia N.	Amino N.
		gm.	*gm.*	*gm.*	*gm.*
I	6	1.166	0.87	0.075	0.021
II	6	2.254	1.318	0.082	0.276
III	12	2.564	1.758	0.122	0.033

Amount of N injected as glycine, *gm*	2.64
" " N recovered " " *gm*	0.25
Injected glycine excreted unchanged, *per cent*	9.5
Amount of extra N in urine resulting from injection (total), *gm*	1.32
Injected N recovered in urine, per cent	50.0

It appears that the excretion of glycine ended during Period II when the urea excretion probably continued high through Period III. The

ammonia excretion was not changed significantly. The percentage of injected glycine which escaped in the urine is one-half of that in Experiment I.

Experiment III.

Injection of Glycine at the Rate of 1 Gm. per Kilo of Body Weight per Hr. for 2 Hrs.

Weight of dog, 16.27 kg. Injected 32.54 gm. of glycine, containing 6.08 gm. of N.

Period I = 3 hrs. Normal control. Before injection.
" II = 2 " Period during which glycine was injected for 2 hrs.
" III = 2 " After period. No injection.

Analysis of Urine.

Period.	Hrs.	Total N.	Urea N.	Ammonia N.	Amino N.
		gm.	*gm.*	*gm.*	*gm.*
I	1, 2, 3	0.468	0.274	0.02	0.039
II	4	0.670	0.253	0.035	0.269
	5	0.834	0.257	0.038	0.478
III	6, 7	1.003	0.621	0.062	0.089

Amount of N injected as glycine, *gm*	6.08
" " N recovered " " *gm*	0.78
Injected glycine excreted unchanged, *per cent*	62.8
Amount of extra N in urine resulting from injection (total), *gm*	1.88
Injected N recovered in urine, *per cent*	30.9

The experiment does not permit the decision that the excretion of unchanged glycine was ended when the experiment closed. It appears to have been still in progress 2 hours after the injection. At this time the urea excretion was still rising.

Experiment IV.

Injection of Glycine at the Rate of 0.75 Gm. per Kilo of Body Weight per Hr. for 1 Hr.

Weight of dog, 11.25 kg. Injected 8.44 gm. of glycine, containing 1.58 gm. of N.

Period I = 6 hrs. Normal control. Before injection.
" II = 6 " Period during which glycine was injected for 1 hr.
" III = 12 " After period. No injection.

Analysis of Urine.

Period.	Hrs.	Total N.	Urea N.	Ammonia N.	Amino N.
		gm.	gm.	gm.	gm.
I	6	0.508	0.380	0.022	0.007
II	6	1.076	0.841	0.055	0.070
III	12	1.527	1.289	0.028	0.080

Amount of N injected as glycine, *gm*..... 1.58
" " N recovered " " *gm*..... 0.13
Injected glycine excreted unchanged, *per cent*..... 8.23
Amount of extra N in urine resulting from injection (total), *gm*..... 1.08
Injected N recovered in urine, *per cent*..... 68.3

The glycine and urea excretions were apparently still in progress at the end of Period III.

Experiment V.

Injection of Glycine at the Rate of 0.5 Gm. per Kilo of Body Weight per Hr. for 3 Hrs.

Weight of dog, 15.22 kg. Injected 22.83 gm. of glycine, containing 4.28 gm. of N.

Period I = 3 hrs. Normal control. Before injection.
" II = 3 " Period during which glycine was injected.
" III = 3 " After period. No injection.

Analysis of Urine.

Period.	Hrs.	Total N.	Urea N.	Ammonia N.	Amino N.
		gm.	gm.	gm.	gm.
I	1, 2, 3	0.491	0.421	0.02	0.026
II	4	0.503	0.394	0.019	0.012
	5	0.873	0.738	0.02	0.026
	6	1.029	0.745	0.029	0.066
III	7, 8, 9	2.167	1.738	0.061	0.047

Amount of N injected as glycine, *gm*..... 4.28
" " N recovered " " *gm*..... 0.10
Injected glycine excreted unchanged, per cent..... 2.33
Amount of extra N in urine resulting from injection (total), *gm*..... 3.1
Injected N recovered in urine, *per cent*..... 72.43

The excess of amino N excretion had ceased at the close of Period III. The urea N had decreased but was not as yet equal to basal excretion. No significant change in ammonia N.

Experiment VI.

Injection of Glycine at the Rate of 0.2 Gm. per Kilo of Body Weight per Hr. for 7 Hrs.

Weight of dog, 14.75 kg. Injected 20.65 gm. of glycine, containing 3.86 gm. of N.

Period I = 2 hrs. Normal control. Before injection.
" II = 7 " Period during which glycine was injected.

Analysis of Urine.

Period.	Hrs.	Total N.	Urea N.	Ammonia N.	Amino N.
		gm.	*gm.*	*gm.*	*gm.*
I	1, 2	0.299	0.169	0.014	0.03
II	3	0.305	0.151	0.015	0.023
	4	0.342	0.208	0.018	0.02
	5	0.431	0.299	0.01	0.015
	6	0.555	0.389	0.014	0.014
	7	0.474	0.344	0.008	0.022
	8	0.599	0.455	0.016	0.016
	9	0.573	0.417	0.02	0.017

Amount of N injected as glycine, *gm.*	3.86
" " N recovered " " *gm.*	0.022
Injected glycine excreted unchanged, *per cent.*	0.57
Amount of extra N in urine resulting from injection (total), *gm.*	2.23
Injected N recovered in urine, *per cent.*	57.8

It looks here as if the 0.2 gm. rate barely overtopped the tolerance limit if it did at all. The excess amino N in urine in 3rd and 4th hours is not greater than the limits of error in the methods employed. After that there was no excess whatever.

Experiment VII.

Injection of Glycine at the Rate of 0.1 Gm. per Kilo of Body Weight per Hr. for 7 Hrs.

Weight of dog, 9.08 kg. Injected 6.36 gm. of glycine, containing 1.19 gm. of N.

Period I = 2 hrs. Normal control. Before injection.
" II = 7 " Period during which glycine was injected.

Analysis of Urine.

Period.	Hrs.	Total N.	Urea N.	Ammonia N.	Amino N.
		gm.	*gm.*	*gm.*	*gm.*
I	1, 2	0.195	0.159	0.006	0.011
II	3	0.13	0.081	0.003	0
	4	0.17	0.109	0.005	0
	5	0.133	0.087	0.003	0
	6	0.126	0.083	0.0	0
	7	0.162	0.067	0.0	0
	8	0.199	0.071	0.003	0
	9	0.36	0.112	0.002	0

Amount of N injected as glycine, *gm*	1.19
" " N recovered " " *gm*	0
Injected glycine excreted unchanged, *per cent*	0
Amount of extra N in urine resulting from injection (total), *gm*	0.6
Injected N recovered in urine, *per cent*	50.4

This is a distinctly subtolerant rate of glycine injection. The urea excretion is still high at the end. Ammonia excretion shows no marked changes.

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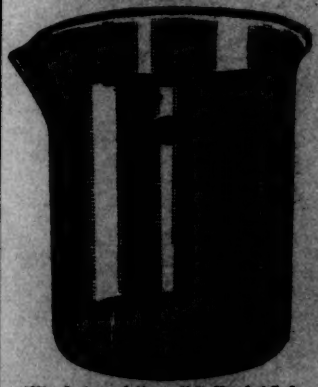

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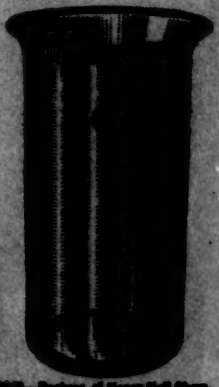

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